

Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts

S. Blank¹, H. Seismann¹, Y. Michel¹, M. McIntyre², L. Cifuentes², I. Braren³, T. Grunwald⁴, U. Darsow^{2,5}, J. Ring^{2,5}, R. Bredehorst¹, M. Ollert² & E. Spillner¹

¹Institute of Biochemistry and Molecular Biology, University of Hamburg; ²Clinical Research Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich; ³Hamburg Center for Experimental Therapy Research (HEXT), University Medical Center Hamburg-Eppendorf, Hamburg; ⁴PLS-Design GmbH, Hamburg; ⁵Division of Environmental Dermatology and Allergy, Helmholtz Center/TUM, Munich, Germany

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Correspondence

Edzard Spillner, Institute of Biochemistry and Molecular Biology, Department of Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany.

Tel.: ++49 40 42838 6982

Fax: ++49 40 42838 7255

E-mail: spillner@chemie.uni-hamburg.de

Markus Ollert, Clinical Research Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy, Biederstein TUM, Biedersteiner Str. 29, 80802 Munich, Germany

Tel.: ++49 89 4140 3550

Fax: ++49 89 4140 3552

E-mail: ollert@lrz.tum.de

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Abstract

Background: Generalized systemic reactions to stinging hymenoptera venom constitute a potentially fatal condition in venom-allergic individuals. Hence, the identification and characterization of all allergens is imperative for improvement of diagnosis and design of effective immunotherapeutic approaches. Our aim was the immunochemical characterization of the carbohydrate-rich protein Api m 10, an *Apis mellifera* venom component and putative allergen, with focus on the relevance of glycosylation. Furthermore, the presence of Api m 10 in honeybee venom (HBV) and licensed venom immunotherapy preparations was addressed.

Methods: Api m 10 was produced as soluble, aglycosylated protein in *Escherichia coli* and as differentially glycosylated protein providing a varying degree of fucosylation in insect cells. IgE reactivity and basophil activation of allergic patients were analyzed. For detection of Api m 10 in different venom preparations, a monoclonal human IgE antibody was generated.

Results: Both, the aglycosylated and the glycosylated variant of Api m 10 devoid of cross-reactive carbohydrate determinants (CCD), exhibited IgE reactivity with approximately 50% of HBV-sensitized patients. A corresponding reactivity could be documented for the activation of basophils. Although the detection of the native protein in crude HBV suggested content comparable to other relevant allergens, three therapeutical HBV extracts lacked detectable amounts of this component.

Conclusion: Api m 10 is a genuine allergen of *A. mellifera* venom with IgE sensitizing potential in a significant fraction of allergic patients independent of CCD reactivity. Thus, Api m 10 could become a key element for component-resolved diagnostic tests and improved immunotherapeutic approaches in hymenoptera venom allergy.

Abbreviations

AP, alkaline phosphatase; CBD, chitin binding domain; CCD, cross-reactive carbohydrate determinant; HBV, honeybee venom; MS, mass spectrometry; NTA, nitrilo-triacetic acid; sIgE, specific IgE; VIT, venom immunotherapy; YJV, yellow jacket venom.

Although venom immunotherapy (VIT) is an effective treatment in the majority of patients, 10–20% of patients were not protected by honeybee venom (HBV) immunotherapy (1, 2). Thus, there is considerable interest in improving diagnosis as well as design, safety, and efficacy of therapy.

The most prominent HBV allergens include phospholipase A₂ (Api m 1), hyaluronidase (Api m 2), and the basic peptide

melittin (Api m 4) (3), all constituting medium to high abundance proteins of HBV (4). Nevertheless, hymenoptera venoms comprise a more complex cocktail of different compounds all of which may contribute to sensitization, symptoms, and success of VIT. Significant progress, however, has been made in the recent years to identify additional compounds of lower abundance, such as the acid phosphatase Api m 3 (5) and the DPPIV enzyme Api m 5 (6).

Recombinant approaches are imperative for the assessment of allergenicity and clinical relevance of such venom compounds, whereby expression should meet the requirements of proper folding and correct posttranslational modifications being potentially important for the establishment of conformational epitopes (7). In particular, glycan structures can contribute significantly to characteristics of venom proteins and constitute the underlying principle of cross-reactive carbohydrate determinants (CCD), a peculiarity of hymenoptera and plant allergens interfering with diagnosis and design of therapeutic strategies (8–10).

A venom protein of considerable interest within this context is Api m 10, the carbohydrate-rich protein. Peptides of this protein were identified by two independent groups in 2005 (11, 12). Insoluble, nonglycosylated protein obtained from *Escherichia coli* exhibited IgE reactivity (13) and an inherent molecular lability. However, without established recombinant expression of soluble Api m 10, its relative abundance in whole HBV as well as its relevance in the context of sensitization and VIT remained elusive.

In this study, we comparatively analyzed differentially glycosylated Api m 10 proteins for their IgE reactivity and basophil activation. The obtained data suggest a pivotal role of this protein as sensitizing agent in HBV allergy, thus supporting its status as a major allergen of clinical relevance in HBV. Furthermore, a monoclonal human IgE antibody allowed quantification of Api m 10 in *Apis mellifera* venom and also demonstrated the absence of this putatively essential component in widely used therapeutic preparations.

Materials and methods

Sera

Three groups of sera from hymenoptera venom-sensitized patients were selected: (i) sera with negative specific IgE (sIgE) test to vespid venom ($i3 < 0.35$ kU/l) but positive test to HBV ($i1 > 0.35$ kU/l) ($n = 17$); (ii) sera with negative sIgE test to HBV ($i1 < 0.35$ kU/l) but positive test to vespid venom ($i3 > 0.35$ kU/l) ($n = 16$); and (iii) sera with positive sIgE test to both ($i1$ and $i3 > 0.35$ kU/l) ($n = 51$). Beekeepers were recruited during daily clinical practice. All patients had given their informed written consent to draw an additional serum sample. The characterization of venom-allergic patients is described in detail in the Data S1.

Cloning of cDNA

Total RNA was isolated from the separated stinger with attached venom sack and glands of honeybee (*A. mellifera*

carnica) using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III RT (Invitrogen, Karlsruhe, Germany) and the gene-specific primer 5'-TCAAG CAGTTAATACATCTCCTTGG-3' were used to synthesize cDNA from the isolated total RNA. Api m 10 cDNA was amplified using *Pfu* DNA polymerase (Fermentas, St. Leon-Roth, Germany) and the primers 5'-TTCCTGGTGCACAC GATGAGG-3' and 5'-TCAAGCAGTTAATACATCTCCTT GG-3'. Cloning and expression in insect cells and *E. coli* is described in detail in the Data S1.

Immunoreactivity of patient sera with recombinant proteins

Specific IgE, IgG, IgG1, and IgG4 immunoreactivity of human sera from hymenoptera venom-allergic patients as well as from beekeepers with purified recombinant proteins (20 µg/ml) was assessed by ELISA. A detailed description of the ELISA is given in the Data S1. The lower end functional cutoff indicated as lines was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cutoff were excluded.

Statistical analysis

Statistical analysis was performed with Prism 3.0 software (GraphPad Software, San Diego, CA, USA). Correlation coefficients were calculated using Pearson correlation analyses.

Basophil activation test

The basophil activation test was performed as described previously (6, 14). A brief description is given in the Data S1.

Other methods

SDS-PAGE and Western blotting are described in detail in the Data S1. Molecular biology standard procedures such as PCR, DNA restriction, ligation, transformation, and plasmid isolation were performed according to established protocols (15). The chimeric human monoclonal IgE antibodies were generated essentially as described recently (16).

Results

Recombinant expression and characterization of Api m 10

For recombinant expression of Api m 10, a putative venom allergen with a theoretical mass of 22 kDa (11, 12), the cDNA of splicing variant 2, was amplified from venom gland cDNA (Fig. S1).

Expression in *E. coli* as aglycosylated protein (17) yielded soluble Api m 10 with an apparent molecular weight of approximately 35 kDa (Fig. 1A, B), suggesting a modified migration behavior because of its low pI. Glycosylated Api m 10 with or devoid of CCDs was produced by baculovirus infection of *Trichoplusia ni* (HighFive) or *Spodoptera frugiperda* (Sf9) insect cells. Purification yielded recombinant Api m 10 with an apparent molecular mass of approximately

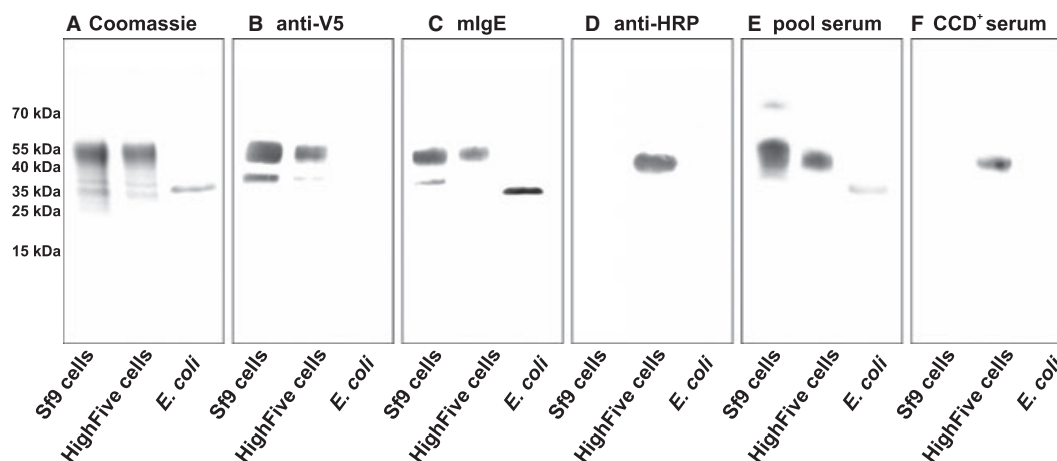


Figure 1 Immunoreactivity of recombinant Api m 10 in Western blot. SDS-PAGE and immunoblot analysis of Api m 10 recombinantly produced in Sf9 and HighFive insect cells as well as in *Escherichia coli* visualized by either Coomassie blue staining or anti-V5

epitope antibody, monoclonal human anti-Api m 10 IgE antibody, anti-HRP antiserum, pooled sera of five honeybee venom-allergic patients, and a cross-reactive carbohydrate determinant-positive serum.

50–55 kDa (Fig. 1A, B) underlining the contribution of the carbohydrates to the overall characteristics.

In immunoblot, all three proteins were reactive with a monoclonal anti-Api m 10 IgE and a serum pool of HBV-sensitized patients (Fig. 1C, E). The use of an anti-HRP rabbit serum specific for α -1,3-core fucosyl residues verified pronounced CCD-based cross-reactivity for Api m 10 produced in HighFive cells. In contrast, glycosylated, Sf9-produced as well as *E. coli*-derived Api m 10 did not exhibit any CCD reactivity (Fig. 1D). Comparable results were obtained with serum of a CCD-reactive but not HBV-allergic patient (Fig. 1F). ELISA analyses corroborated the data obtained in immunoblotting regarding protein identity and presence of CCDs (Fig. 2). These data demonstrate that the host defines the state of gly-

cosylation (18) and, thereby, strongly influences the characteristics of the allergens.

Screening of patient sera for IgE reactivity with Api m 10 variants

Sera of 84 randomly selected patients with a clinical history of insect venom allergy (Table S1) were separated into three groups and assayed by ELISA for sIgE antibodies to Api m 10 produced in Sf9 insect cells.

In group I of 51 sera double positive for HBV and yellow jacket venom (YJV), thus, predominantly cross-reactive, 27 sera (52%) exhibited pronounced reactivity with Sf9-derived Api m 10 (Fig. 3A). From group II of 17 sera with negative

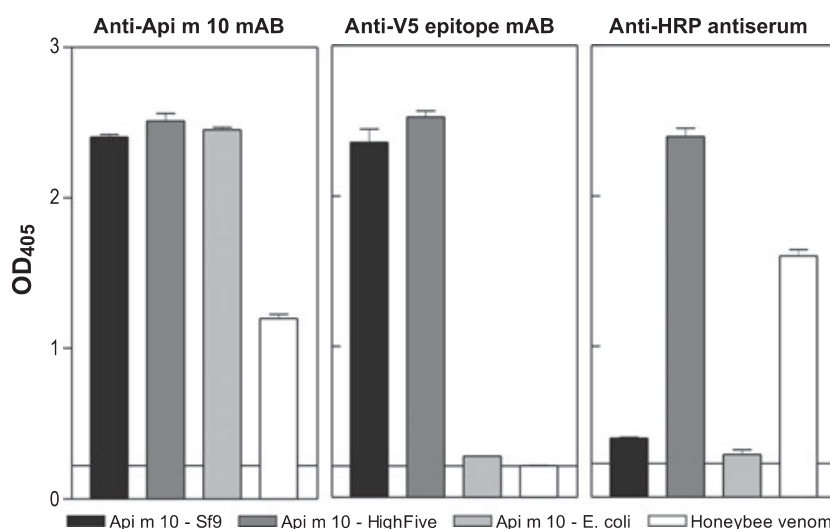


Figure 2 Immunoreactivity of recombinant Api m 10 in ELISA. ELISA analysis of Api m 10 and honeybee venom using the mono-

clonal anti-Api m 10 IgE, the anti-V5 epitope antibody, and the anti-HRP antiserum. Results are presented as triplicates.

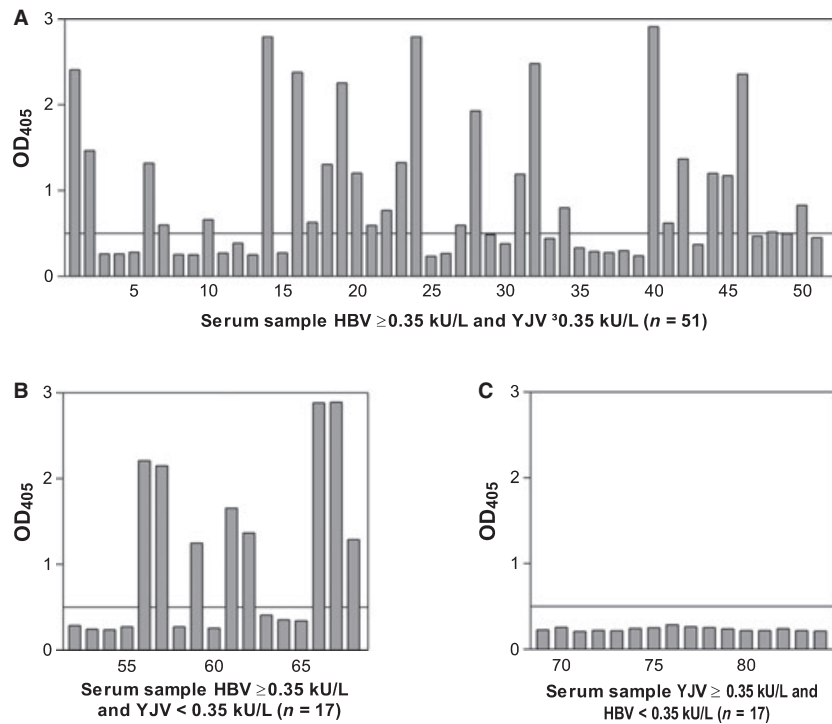


Figure 3 IgE immunoreactivity of individual patient sera with recombinant Api m 10 produced in Sf9 insect cells. The IgE reactivity was assessed by ELISA with 17 sera of venom-sensitized

patients with negative IgE to vespid venom (A), 16 sera of venom-sensitized patients with negative IgE to *Apis mellifera* venom (B), and 51 double-positive sera (C).

sIgE to YJV implying sensitization to HBV only without CCD reactivity, 8 sera (47%) reacted with Api m 10 (Fig. 3 B). In group III of 16 sera with negative sIgE to HBV but positive sIgE to YJV, none of the sera recognized Api m 10, suggesting the absence of a cross-reactive molecule in YJV (Fig. 3C). As anticipated, the presence of CCDs upon production of Api m 10 in HighFive insect cells resulted in augmented IgE reactivities in group I (data not shown).

In comparative assessment of sIgE binding to Sf9- and *E. coli*-derived Api m 10, in group I, 18/38 (47%) and 17/38 (44%), respectively, exhibited pronounced reactivity with the protein variants (Fig. 4A), and in group II 5/11 sera (45%) (Fig. 4B). Group III (Fig. 4C) exhibited no reactivity at all. Reactivities of selected sera found positive in ELISA (patients 16, 19, and 24; patient 69 as control) were further verified in immunoblot (Fig. 4E).

Moreover, sera of beekeepers as well as of venom-allergic patients were investigated for Api m 10- and Api m 1-specific IgG1, IgG4, and IgE antibody responses (Fig. S3). Thereby, over 50% of beekeepers showed relevant IgG1 and IgG4 immune responses to Api m 10, a picture that although less pronounced was also evident for the venom-allergic patients.

Activation of basophils from venom-allergic patients

The capability of Api m 10 produced in HighFive insect cells or *E. coli* for activation of human basophils was assessed and related to that of established major allergens, Api m 1 (or Ves v 5). All blood samples were obtained from consecutively

selected patients ($n = 15$) (see Table S2) with a clinical history of a severe reaction after a stinging event (13 with honeybee as relevant insect and two additional patients with yellow jacket as relevant insect). All patients had a positive intradermal skin test and sIgE test for HBV and/or YJV.

Overall, 8/13 patients with honeybee as relevant insect showed positive basophil activation with Api m 10. With the species-specific major allergen Api m 1 or Ves v 5, 14/15 patients had positive basophil activation. Figure 5A–F depicts representative results for six exemplary patients. Patients 1, 8, and 10 showed comparable basophil activation (Fig. 5A, C, D) for Api m 10 and Api m 1, patient 6 (Fig. 5 B) to a varying degree. Patient 11 exhibited activation by Api m 1 exclusively (Fig. 5E). Patient 15 (Fig. 5F) exhibited activation for Api m 10 and strongly reduced for Api m 1, a picture fully reflected in the sIgE titers. Interestingly, the basophil activation was comparable for the aglycosylated and the glycosylated protein.

Together, these results show that Api m 10 is able to induce relevant effector cell activation and thus has to be considered as an important allergen in *A. mellifera* venom. Moreover, carbohydrates appear to contribute to the overall IgE reactivity to a minor extent only.

Evaluation of native Api m 10 in *Apis mellifera* venom

To clarify the molecular integrity and concentration of Api m 10 in the native HBV and in preparations used for VIT, a monoclonal human IgE antibody was generated (Fig. S2),

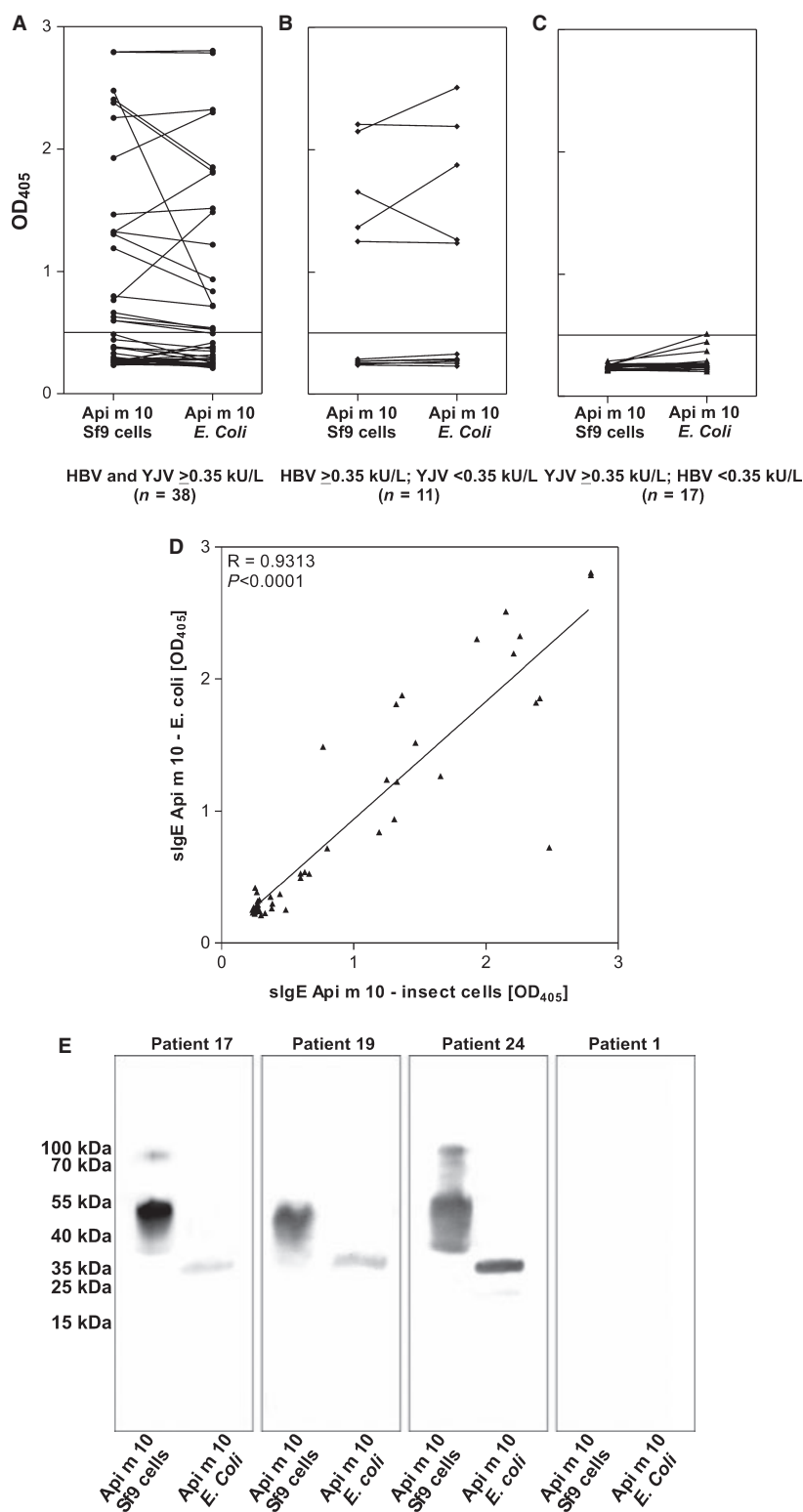


Figure 4 IgE immunoreactivity of individual patient sera with Api m 10 produced in Sf9 insect cells and *Escherichia coli*. The IgE reactivity was assessed with 38 double-positive sera (A), 11 sera with negative IgE to vespid venom (B), and 16 sera with nega-

tive IgE to *Apis mellifera* venom (C). Correlation was assessed by Pearson correlation analysis (D). In parallel, the reactivity of four particular sera with recombinant Api m 10 was assessed in immunoblot (E).

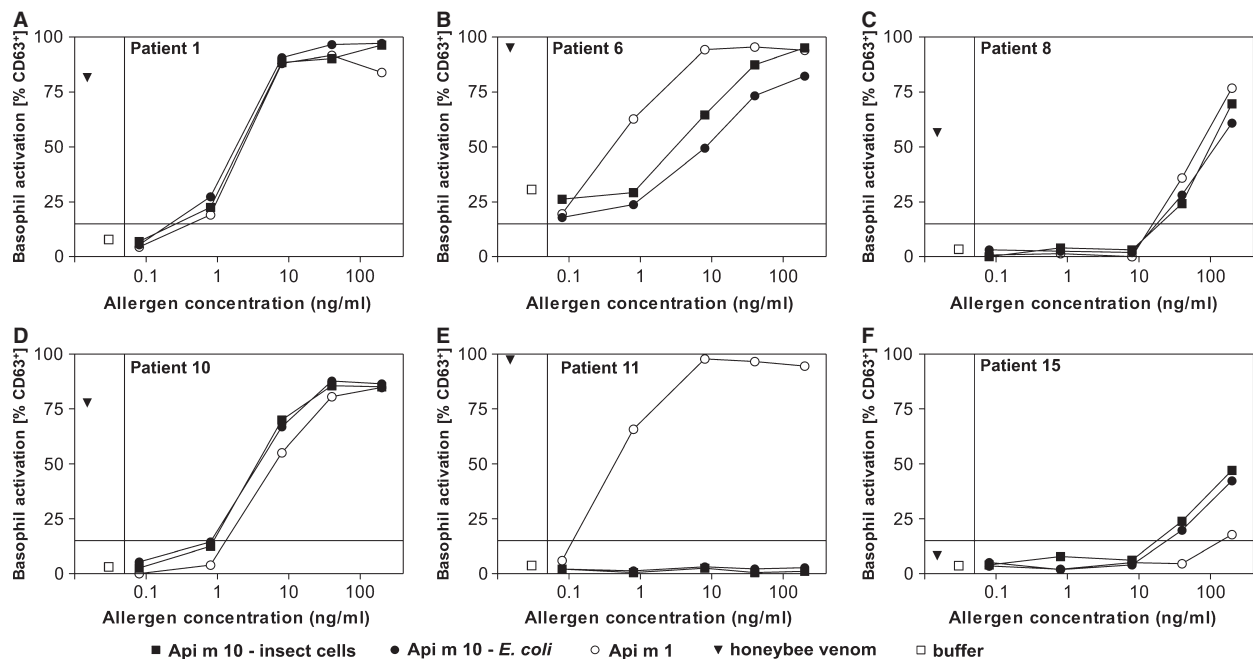


Figure 5 Basophil activation tests with recombinant Api m 10. Human basophils from six exemplary honeybee venom (HBV)-sensitized patients were exposed to serial dilutions of Api m 10 produced in either insect cells (filled squares) or *Escherichia coli* (filled circles) and Api m 1 as established reference allergen (open circles). Additionally, stimulation with HBV (filled triangles) and plain stimulation buffer (open circles) is shown. Activation is shown as percentage of CD63⁺ cells.

the reactivity of which with Api m 10 was verified in ELISA and immunoblot (Figs 1C and 2A). A human monoclonal IgE antibody with specificity for acid phosphatase (Api m 3) was applied analogously (Fig. 6A, lower panel).

Applying this antibody in immunoblots of crude HBV, a major band at 55 kDa was detected (Fig. 6A, upper panel). To address the quantity of Api m 10 in HBV, the monoclonal IgE was applied to immunoblots providing serial dilutions of recombinant Sf9-derived Api m 10 and HBV (Fig. 6A, upper panel). Densitometric quantification sug-

gested a concentration in the range of $8 \pm 1 \mu\text{g}$ per g of crude HBV, corresponding to $0.8\% \pm 0.1\%$ of dry weight compared with $1.7\% \pm 0.4\%$ for Api m 3.

Additionally, crude venom and three different HBV preparations for VIT from different allergen extract producers were analyzed (Fig. 6B, left panel). Api m 1 was used as a control for loading equal amounts (Fig. 6B, lower panel). In stark contrast to the crude venom, no reactivities at all were obtained for any of the three preparations. Api m 3 could be readily detected in whole HBV as well as in two of the

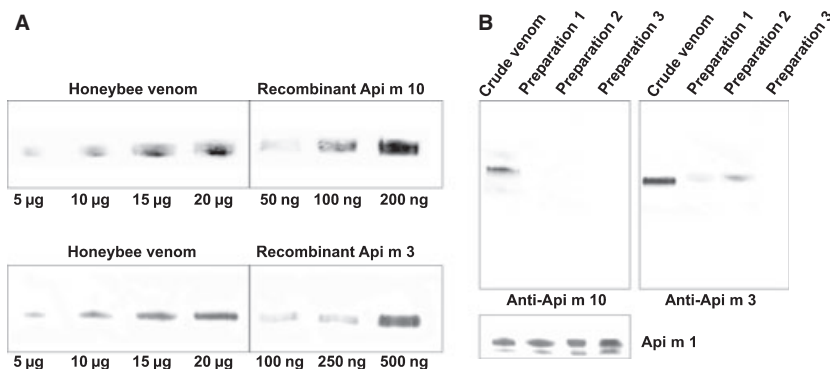


Figure 6 Quantification of Api m 10 in honeybee venom (HBV) and therapeutic venom extracts. Concentration of Api m 10 in HBV was assessed by densitometric analyses of serial dilutions of recombinant Api m 10 and Api m 3 and in crude venom by immunoblotting using

an anti-Api m 3 and an anti-Api m 10 IgE antibody (A). The presence of Api m 10 and Api m 3 in crude venom and venom preparations (each 25 μg per lane, Coomassie staining of Api m 1 was employed as additional loading control) was addressed analogously (B).

preparations, although in significantly lesser amounts (Fig. 6 B, right panel). These data demonstrate that the concentrations of the putatively labile HBV allergen Api m 10 and the already established and classical allergen Api m 3 are dramatically reduced in therapeutic venom preparations investigated in this study.

Discussion

In the last decades, much effort has been spent to characterize a plethora of allergens in a variety of sources. However, hymenoptera venoms as the allergenic source causing the highest incidence of anaphylaxis and sometimes even fatal consequences remain inadequately characterized with regard to their molecular composition. Despite the fact that the higher abundance allergens of HBV (Api m 1, Api m 2, and Api m 4) have already been characterized in detail years ago, comparatively little is known about the sensitizing potential, the allergenicity, and the clinical relevance of the lower abundance allergens. One of these putative allergens is Api m 10, a protein of unknown function, peptides of which were recently identified in HBV by two independent groups (11, 12).

Contrary to a previous report (13), we readily obtained soluble recombinant Api m 10 using both the eukaryotic baculovirus expression system or the strategy of chitin binding domain fusion followed by autocatalytic intein-mediated cleavage in *E. coli*. The difference of molecular weight between the aglycosylated and the insect cell-derived, glycosylated proteins obviously stems from extensive posttranslational modifications, such as glycosylation.

It has increasingly been recognized that cross-reactivities based on glycosylation, namely by alpha-1,3-core fucose residues of *N*-glycans, represent a major concern for diagnostic approaches in hymenoptera venom allergy. In this regard, the use of Sf9 insect cell lines constitutes a strategy recently reported by us to circumvent the establishment of CCDs (18) under the aegis of an autologous eukaryotic expression. Further support for this concept becomes evident by the augmented reactivities of the HighFive cell-produced Api m 10 displaying CCDs (see Figs 1 and 2).

Using the differentially glycosylated protein variants, up to approximately 50% of HBV-allergic patients showed reactivity in ELISA and basophil activation tests with recombinant Api m 10 beyond CCD reactivity, thus rendering it an important sensitizing component of HBV. This finding was supported by a pronounced IgG4 immune response in over 50% of beekeeper sera. Induction of IgG4 is an intrinsic potential of all allergens known to be good inducers of IgE, as demonstrated for Api m 1 and other relevant allergens (19). Interestingly, the findings obtained with the aglycosylated protein expressed in *E. coli* matched those with the glycosylated protein. The complete lack of reactivity in the group of YJV-sensitized patients without sensitization to HBV additionally suggests the absence of a homolog in vespid venom rendering Api m 10 a genuine marker for HBV allergy.

As evident from the data of this study, Api m 10 appears to be a crucial but delicate component of HBV preparations.

By the use of the monoclonal anti-Api m 10 IgE, we were for the first time able to detect native Api m 10 in unprocessed HBV. It is present as intact protein with concentrations (0.8%) only slightly lower than those of Api m 3 (1.7%) and Api m 2 (2–3%) (20). Thus, it was surprising that Api m 10 is apparently absent or at least vastly underrepresented in three therapeutic preparations of HBV from independent vendors. Obviously, downstream processing of venoms for VIT affects the distribution of venom proteins, resulting in the loss of particular low abundance components.

While the exact role of lower abundance allergens for therapeutic efficacy of VIT with HBV remains to be analyzed, the presence of lower abundance or inherently labile venom components should become imperative as proof of extract quality.

In summary, Api m 10 from *A. mellifera* venom for the first time could be produced in soluble form in baculovirus-infected insect cells as well as in *E. coli*. The results obtained with these proteins suggest an important role for Api m 10 as sIgE-sensitizing component in HBV allergy beyond its carbohydrate-based cross-reactivity and emphasize its clinical relevance. This relevance will additionally be verified in future studies on the relevance of different recombinant venom allergens for therapeutic efficacy. Results of such studies might help to elucidate the needs and pitfalls of successful VIT.

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Author contributions

SB, HS, YM, and IB performed cloning and expression; SB, HS, IB, MO, and ES involved in manuscript preparation; MM carried out basophil activation tests; CL and DU involved in recruitment of beekeepers; TG, JR, and RB analyzed the data; and MO and ES involved in study design and analysis.

Conflicts of interest

The authors declare that they have no financial, research, organizational, or other interests to disclose that are relevant to the execution of this research or this publication.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Figure S1. Alignment of Api m 10 variants.

Figure S2. Generation and expression of a monoclonal human IgE by selection of a combinatorial library.

Figure S3. Serological characterization of beekeepers and patients assessed in basophil activation test.

Data S1. Supplementary materials and methods.

Table S1. Serological data of patients assessed in IgE reactivity analysis.

Table S2. Characterization of the venom-allergic patients assessed in basophil activation test.

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Supplementary materials and methods

Materials

Crude HBV as well honeybee phospholipase A2 were purchased from Latoxan (Valence, France). Therapeutic grade HBV preparations were obtained from three different manufacturers. Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG AP conjugate and anti-mouse IgG AP conjugate were obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). Monoclonal AP conjugated monoclonal anti-IgG1 antibody was from Acris Antibodies (Herford, Germany) and AP conjugated monoclonal anti-IgG4 was from Siemens Healthcare Diagnostics (Los Angeles, CA).

Protein biochemistry

400 µg of *A. mellifera* venom dissolved in 30 µl 5x PAGE loading dye were subjected to SDS-PAGE. Bands in the range of 35 kDa were excised, the proteins digested in-gel by trypsin (Roche Diagnostics, Penzberg, Germany) and resulting peptide fragments were sequenced on a Waters Micromass QToF2 mass spectrometer (Waters, Milford, MA, USA) by tandem mass spectrometry according to the manufacturer's instructions.

Cloning and expression of Api m 10 in insect cells

Subcloned Api m 10 cDNA was used for secondary amplification of the coding region using *Pfu* DNA polymerase in two consecutive PCR reactions adding a N-terminal 10-fold His-tag and V5 epitope using the primers 5'-AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG TGG CTC GAG TCT AGA TTC CCT GGT GCA CAC GAT GAG G-3', 5'-GAT CGG ATC CCA TCA CCA CCA CCA TCA TCA CCA CCA CCA TTC TTC TGG TGG TAA GCC TAT CCC TAA CCC TCT CCT CGG-3' and 5'-GAT CGC GGC

CGC TCA AGC AGT TAA TAC ATC TCC TTG G-3'. The PCR product was subcloned via BamHI and NotI into the digested baculovirus transfer vector pAcGP67-B (BD Pharmingen, Heidelberg, Germany).

Recombinant baculovirus production

Spodoptera frugiperda cells (Sf9) (Invitrogen, Karlsruhe, Germany) were grown at 27 °C in serum-free medium (Express Five SFM; Lonza, Verviers, Belgium containing 10 µg/ml gentamycin; Invitrogen, Karlsruhe, Germany). Cell density was determined by haemocytometer counts, cell viability was evaluated by staining with Trypan Blue. Recombinant baculovirus was generated by cotransfection of Sf9 cells with BaculoGold bright DNA (BD Pharmingen, Heidelberg, Germany) and the baculovirus transfer vector pAC-GP67-B containing Api m 10. High titer stocks were produced by three rounds of virus amplification and optimal MOI for protein expression was determined empirically by infection of Sf9 cells in 100 ml suspension flasks ($1.5\text{--}2 \times 10^6$ cells/ml in 20 ml suspension culture) with serial dilutions of high titer virus stock.

Expression in baculovirus-infected insect cells

High titer stock of recombinant baculovirus was used to infect 400 ml suspension culture of Sf9 or HighFive cells (Invitrogen, Karlsruhe, Germany) ($1.5\text{--}2.0 \times 10^6$ cells per ml) in 2000 ml flasks. For protein production the cells were incubated at 27 °C and 110 rpm for 72 h.

Protein purification

The supernatant of baculovirus-infected cells was collected, adjusted to pH 8, centrifuged at 4000 x g for 5 minutes, and applied to a nickel-chelating affinity matrix (NTA-agarose, Qiagen, Hilden, Germany). The column was washed with binding buffer (50 mM

sodium phosphate, pH 7.6, 500 mM NaCl) and pre-eluted with NTA binding buffer containing 20 mM imidazole. The recombinant protein was eluted from the matrix using NTA-binding buffer containing 300 mM imidazole. Purification was confirmed by SDS-PAGE.

Recombinant bacterial expression and purification of Api m 10

For expression in *E. coli* the Api m 10 coding region was amplified using the primers 5'-GAT CCA TAT GTT CCC TGG TGC ACA CGA TG-3' and 5'-GGT GGT TGC TCT TCC GCA AGC AGT TAA TAC ATC TCC TTG G-3' and inserted into the digested vector pTXB1 (New England Biolabs, Bad Schwalbach, Germany) via NdeI and SapI. The vector was further modified by addition of a second chitin binding domain (CBD). Expression and purification of the fusion protein was performed according to the recommendations of the manufacturer.

Western blotting

For immunoblot procedures the purified recombinant allergens, the crude venom, as well as the therapeutic venom preparations were separated by SDS-PAGE and immobilized onto nitrocellulose membranes. Human sera were diluted 1:10 with 5 mg/ml BSA in PBS and applied to the corresponding Western blots. Visualization of bound IgE was then performed with anti-human IgE mAb conjugated to alkaline phosphatase and nitroterazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate according to the recommendations of the manufacturer. The recombinant monoclonal IgE antibodies were diluted using DMEM supplemented with 100 mL/L heat-inactivated fetal calf serum and applied to the corresponding Western blots and visualized as described above. For Western blot procedures with anti-V5 epitope mAb and anti-HRP antiserum the antibodies were applied according to the recommendations

of the manufacturer and bound antibodies visualized via corresponding secondary antibodies conjugated to alkaline phosphatase as described above.

Immunoreactivity of patient sera with recombinant proteins

For assessment of specific IgE, IgG, IgG1 and IgG4 immunoreactivity of human sera in ELISA, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with purified recombinant proteins (20 µg/ml) at 4 °C overnight and blocked with 40 mg/ml milkpowder in PBS. Thereafter, human sera were diluted 1:2 with PBS and incubated in a final volume of 20 µl for 4 hours at room temperature. After washing 4 times with PBS bound IgE, IgG, IgG1 or IgG4 were detected with a monoclonal alkaline phosphatase-conjugated anti-human IgE, IgG, IgG1 or IgG4 antibody (BD Pharmingen, Heidelberg, Germany) diluted 1:1000. After washing 4 times with PBS 50 µl of substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) per well were added. The plates were read at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cut-off were excluded.

Basophil activation test

The basophil activation test was performed as described previously (1, 2) with modifications as recommended by the manufacturer (Flow-CAST; Bühlmann Laboratories, Basel, Switzerland). In brief, within 3 h after sampling of patient blood, aliquots of 50 µl whole blood were preincubated for 10 min at 37°C with stimulation buffer containing IL-3. Subsequently, basophils were activated for 40 min at 37°C in a water bath with either native or recombinant allergens at a volume of 100 µl. Thereby, HBV (in a concentration of 50 ng/ml) as well as the allergens (Api m 10 produced in either insect cells or *E. coli* and the established major allergen Api m 1, in a concentration range of 0.08–1000 ng/ml) were

applied in concentrations according to those reported in the literature (3-5). A murine mAb against the human high-affinity FcεR served as a positive stimulation control. Plain stimulation buffer was used as a negative stimulation control. To quantify activated basophils, cells were stained with 20 µl reagent containing a mixture of mAbs to human CD63 labeled with PE (anti-CD63–PE) and human IgE labeled with FITC (anti-IgE–FITC) for 30 min on ice. RBCs were lysed, and WBCs were fixed (BD FACS Lysing Solution; BD Biosciences, San Jose, CA) for 5 min at room temperature. After centrifugation (5 min at 1200 x g), cells were resuspended in 500 µl of stop solution. Flow cytometric analysis of basophil activation was performed on a FACScan flow cytometer (BD Biosciences). IgE-staining and side scatter were employed to gate on at least 500 basophils that expressed a high density of surface IgE. Subsequently, within this gate, the percentage of activated basophils (i.e., coexpressing CD63) was measured.

Characterization of patients

The sIgE levels for honeybee venom (HBV) (i1) and yellow jacket venom (YJV) (i3) were determined with the Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, Ca.) or ImmunoCap 250 (Phadia, Uppsala, Sweden) as described in detail elsewhere (6), and for Api m 10, Api m 1, and the MUXF-HSA conjugate as described in the materials and methods section. For intradermal testing of patients with suspected insect venom allergies serial 10-fold dilutions of venom extracts with concentrations ranging from 0.0001 to 0.1 mg/L were performed. Histamine hydrochloride and physiologic saline were used as positive and negative control solutions, respectively. Intradermal tests were rated positive when the wheal size was >5 mm in diameter with a surrounding erythema.

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Supplementary data Figure 1: Alignment of Api m 10 variants.

Shown are Api m 10 splice variants 1 and 2. Peptides identified by mass spectrometry are highlighted in light grey. Signal sequences are italicized and putative glycosylation sites are represented in dark grey.

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Api m 10 V1  MKTLGVLFIAAWFIACTHSFPGAHDSEDSKEERKNVDTVLVLPSIERDQMMAATFDFPSLS 60
Api m 10 V2  MKTLGVLFIAAWFIACTHSFPGAHDSEDSKEERKNVDTVLVLPSIERDQMMAATFDFPSLS 60
*****

Api m 10 V1  FEDSDEGSNWNWNTLLRPNFLDGWYQTLQSAISAHMKKVREQMAGILSRIPEQGVVWNK 120
Api m 10 V2  FEDSDEGSNWNWNTLLRPNFLDGWYQTLQT---HMKKVREQMAGILSRIPEQGVVWNK 116
*****:*****

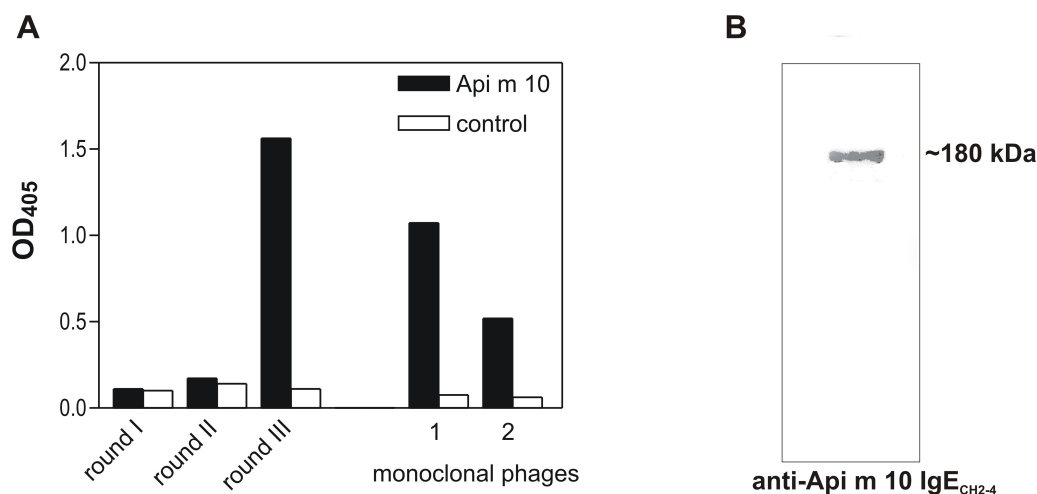
Api m 10 V1  IPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLIRVRVIDVRPQNETILTVSSEAD 180
Api m 10 V2  IPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLIRVRVIDVRPQNETILTVSSEAD 176
*****

Api m 10 V1  SDVTTLPLILIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLTA 223
Api m 10 V2  SDVTTLPLILIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLTA 219
*****

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Supplementary data Figure 2: Generation and expression of a monoclonal human IgE by selection of a combinatorial library.

The immunoreactivity of phage-displayed polyclonal phages from three panning rounds and selected monoclonal phages against Api m 10 was analyzed by ELISA. Controls (white bars) were performed by omission of antigen (A). After conversion of the antibody gene into the format of a monoclonal human IgE antibody, clone 1 was produced in HEK293 cells and detected in cellular supernatant by using monoclonal anti-human IgE-AP conjugate as described in *Material and Methods* (B). Panning procedure and expression of IgE antibody are described elsewhere (1).

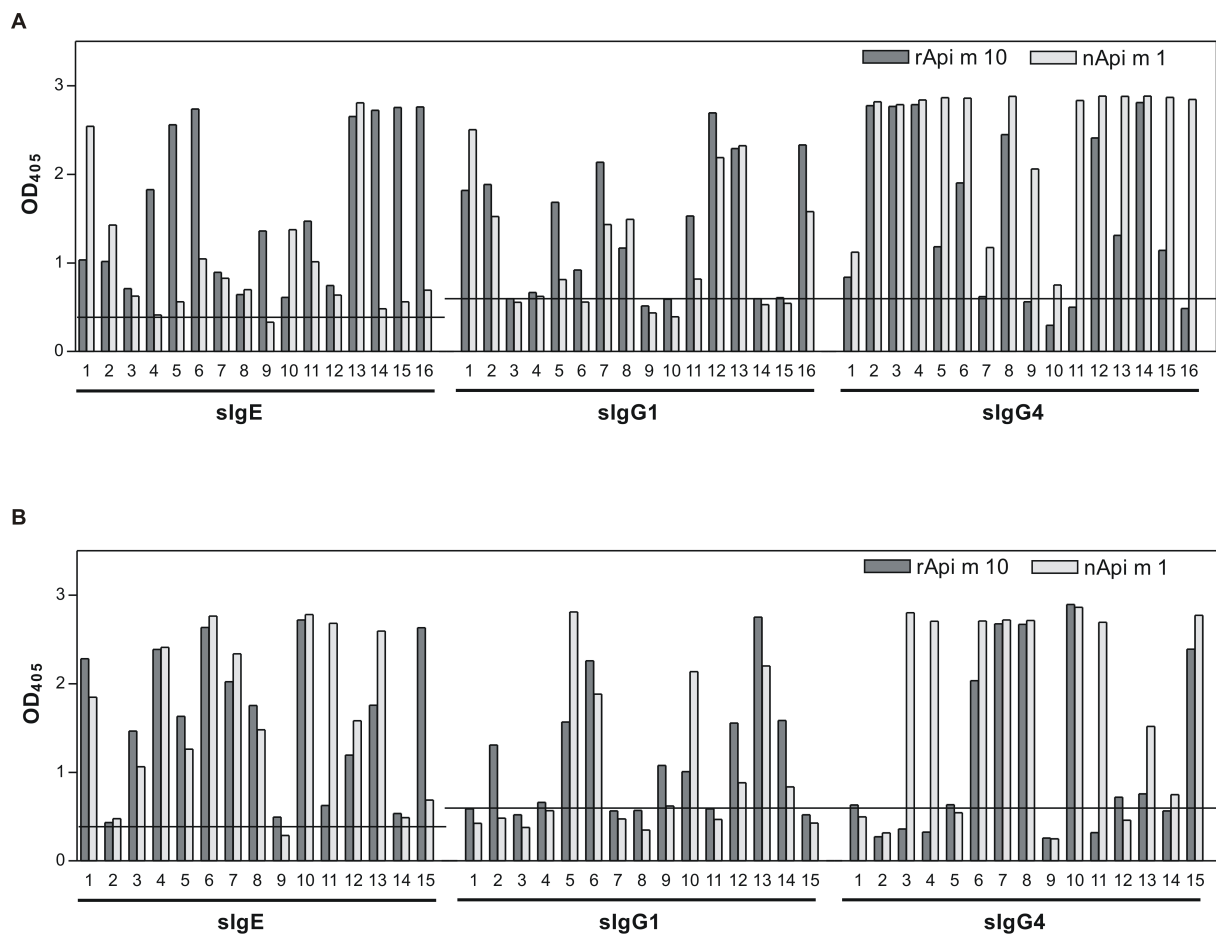


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Supplementary data Figure 3: Serological characterization of beekeepers and patients assessed in basophil activation test.

The sIgE, sIgG1, and sIgG4 levels for rApi m 10 and n Api m 1 of beekeeper sera (A) as well as of sera from hymenoptera venom allergic patients assessed in basophil activation test (B) were determined by ELISA as described in the methods section. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cut-off were excluded.



Supplementary data Table I: Serological data of patients assessed in IgE reactivity analysis

The sIgE levels for honeybee venom (HBV) (i1) and yellow jacket venom (YJV) (i3) were determined with the Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, Ca.) or ImmunoCap 250 (Phadia, Uppsala, Sweden), and for Api m 10 as described for Fig. 3 (negative cutoff: OD<0.5; +: OD $\geq$ 0.5<1; ++: OD $\geq$ 1) . For intradermal testing of patients with suspected insect venom allergies serial 10-fold dilutions of venom extracts with concentrations ranging from 0.0001 to 0.1 mg/L were performed. Histamine hydrochloride and physiologic saline were used as positive and negative control solutions, respectively. Intradermal tests were rated positive when the wheal size was >5 mm in diameter with a surrounding erythema.

patient no.	sIgE Api m 10	sIgE HBV [class]	sIgE HBV [kU/L]	sIgE YJV [class]	sIgE YJV [kU/l]	intradermal skin test (HBV)
1	++	3	6.49	3	14.4	+
2	++	2	0.95	2	1.23	n.d.
3	-	1	0.46	1	0.41	+
4	-	2	1	2	0.73	+
5	-	2	1.61	2	1.71	+
6	++	2	1.43	2	1.4	-
7	+	2	1.2	1	0.446	+
8	-	2	2.28	2	2.33	+
9	-	1	0.5	3	5.37	-
10	+	2	2.23	2	2.36	+
11	-	1	0.635	2	1.21	+
12	-	2	2.97	3	7.99	-
13	-	4	21	2	2.05	+
14	++	6	>100	2	1.16	+
15	-	3	5.2	4	17.9	-
16	++	3	14.1	2	0.81	+
17	+	3	3.54	3	6.75	+
18	++	4	20.5	2	1.42	+
19	++	3	16.6	2	2.17	+
20	++	5	52.8	1	0.41	+
21	+	3	14.8	2	3.07	+
22	+	3	3.86	3	14.1	n.d.
23	++	3	5.54	3	6.76	n.d.
24	++	6	>100	3	6.72	+
25	-	3	4.04	4	33.4	-
26	-	2	0.814	4	18.4	+
27	+	4	9.02	3	9.67	+
28	++	3	13.8	3	8.29	+

29	-	3	11.2	3	11.6	+
30	-	3	4.44	3	9.72	-
31	++	4	21.1	3	4.69	+
32	++	5	56.1	3	8.27	+
33	-	3	4.98	3	15.9	+
34	+	4	21	3	15.5	n.d.
35	-	3	9.93	5	96.2	+
36	-	3	12.6	1	0.562	+
37	-	4	17.7	3	7.65	+
38	-	3	6	5	56	+
39	-	4	20.6	1	0.559	+
40	++	4	22.2	2	2.82	+
41	+	3	10.9	3	11.9	+
42	++	3	12.2	3	3.53	+
43	-	4	24.9	3	13.8	+
44	++	3	5.04	3	8.21	+
45	++	4	23.2	3	5.16	+
46	++	6	>100	4	20.5	+
47	-	3	11.1	3	14.4	+
48	-	3	4.08	3	6.59	+
49	-	4	19.4	4	26.5	+
50	+	4	20.1	3	8.74	n.d.
51	-	4	9.02	3	9.67	+
patient no.	sIgE Api m 10	sIgE HBV [class]	sIgE HBV [kU/L]	sIgE YJV [class]	sIgE YJV [kU/l]	intradermal skin test (HBV)
52	-	2	2.56	0	<0.1	+
53	-	1	0.66	0	<0.1	+
54	-	2	2.11	0	<0.35	+
55	-	2	1.03	0	<0.1	+
56	++	2	1.75	0	<0.1	+
57	++	1	0.69	0	<0.1	+
58	-	1	0.45	0	0.2	+
59	++	2	1.87	0	<0.1	+
60	-	1	0.5	0	<0.1	n.d.
61	++	4	31.6	0	<0.1	+
62	++	3	4.01	0	0.266	+
63	-	2	2.83	0	0.34	+
64	-	2	2.32	0	<0.1	+
65	-	3	3.61	0	<0.1	n.d.
66	++	3	11.8	0	<0.1	n.d.
67	++	3	7.84	0	<0.1	+
68	++	3	4.99	0	0.24	+
patient no.	sIgE Api m 10	sIgE HBV [class]	sIgE HBV [kU/L]	sIgE YJV [class]	sIgE YJV [kU/l]	intradermal skin test (HBV)
69	-	0	<0.1	4	4	-
70	-	0	<0.1	2	3.25	-
71	-	0	<0.1	1	0.461	-
72	-	0	<0.1	3	9.61	-
73	-	0	0.306	4	30.8	-
74	-	0	<0.1	5	75.5	-
75	-	0	0.221	3	16.9	+
76	-	0	<0.1	4	23.5	-
77	-	0	<0.1	4	27	-
78	-	0	<0.1	4	19.4	-
79	-	0	<0.1	3	8.69	-
80	-	0	<0.1	2	1.83	-
81	-	0	<0.1	5	75.6	-
82	-	0	<0.1	3	6.59	-
83	-	0	0.22	4	19.2	-
84	-	0	<0.1	3	8.18	+

Supplementary data Table II: Characterization of the venom-allergic patients assessed in basophil activation test

The sIgE levels for honeybee venom (HBV) (i1) and yellow jacket venom (YJV) (i3) were determined with the Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, Ca.) or ImmunoCap 250 (Phadia, Uppsala, Sweden), and for Api m 10, Api m 1, and the MUXF-HAS conjugate by ELISA as described in the methods section. For intradermal testing of patients with suspected insect venom allergies serial 10-fold dilutions of venom extracts with concentrations ranging from 0.0001 to 0.1 mg/L were performed. Histamine hydrochloride and physiologic saline were used as positive and negative control solutions, respectively. Intradermal tests were rated positive when the wheal size was >5 mm in diameter with a surrounding erythema.

Patient I.D.	Relevant Insect	Anaphylaxis grade ¹	Skin test ²		sIgE – Extract ³		sIgE – Allergens ⁴			
			HBV (µg/ml)	VV (µg/ml)	i1 (kU/L)	i3 (kU/L)	Api m 10 Insect cells (OD _{450nm})	Api m 10 <i>E. coli</i> (OD _{450nm})	Api m 1 (OD _{450nm})	MUXF (OD _{450nm})
1	HB	1	0,001	negative	13,4	1,58	2,382	2,491	2,196	1,299
2	YJ	2	negative	0,01	<0.1	22,1	0,294	0,318	0,247	0,278
3	HB	2	0,0001	negative	60.8	0.521	2,719	2,726	0,896	0,709
4	HB	2	0,001	negative	27.6	<0.1	2,442	2,628	1,744	1,018
5	HB	1	0.1	negative	2.33	<0.1	2,691	2,716	0,908	0,693
6	HB	2	0,0001	negative	23.8	2,19	2,446	2,709	2,547	2,867
7	HB	2	0,0001	negative	72.7	0,124	2,438	2,051	1,824	0,925
8	HB	2	0,01	0,1	3,21	2,17	2,426	2,590	1,301	2,818
9	YJ	2	negative	0,01	<0.1	1,29	0,347	0,306	0,224	0,265
10	HB	2	0,01	negative	81.4	3,31	2,422	2,684	2,733	2,792
11	HB	2	0,0001	0,01	1,89	0,19	0,540	0,528	2,737	0,535
12	HB	1	0,01	0,01	4,32	8,31	2,431	1,706	0,527	1,712
13	HB	2	0,0001	negative	3,26	0,11	1,079	0,895	0,814	0,937
14	HB/YJ	3	0,001	0,001	1.33	0.467	0,516	0,423	0,311	0,453
15	HB	3	0,01	0,01	3,88	0,4	2,428	2,631	0,438	2,248

¹ According to Ring and Messmer (1).

² Shown is the lowest venom extract concentration that gave a positive result in intradermal skin testing applying a ten-fold concentration range from 0.1 to 0.0001 µg/ml for each of the venoms.

³ As tested in either UniCAP250 or Immulite2000 sIgE assays using a lower end cut-off of 0.35 kU/L.

⁴ As tested in an IgE ELISA using a lower end cut-off of 0.55 as described in the methods section.

References

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