Component resolution reveals additional major allergens in patients with honeybee venom allergy☆

Julian Köhler, MS,[a]* Simon Blank, PhD,[b]* Sabine Müller, MD,[a] Frank Bantleon, DiplBiol,[b] Marcel Frick, MS,[a] Johannes Huss-Marp, MD,[a,c] Jonas Lidholm, PhD,[d] Edzard Spillner, PhD,[b]‡ and Thilo Jakob, MD[a]‡ *Freiburg and Hamburg, Germany, and Uppsala, Sweden*

Background: Detection of IgE to recombinant Hymenoptera venom allergens has been suggested to improve the diagnostic precision in Hymenoptera venom allergy. However, the frequency of sensitization to the only available recombinant honeybee venom (HBV) allergen, rApi m 1, in patients with HBV allergy is limited, suggesting that additional HBV allergens might be of relevance.

Objective: We performed an analysis of sensitization profiles of patients with HBV allergy to a panel of HBV allergens.

Methods: Diagnosis of HBV allergy (n = 144) was based on history, skin test results, and allergen-specific IgE levels to HBV. IgE reactivity to 6 HBV allergens devoid of cross-reactive carbohydrate determinants (CCD) was analyzed by ImmunoCAP.

Results: IgE reactivity to rApi m 1, rApi m 2, rApi m 3, nApi m 4, rApi m 5, and rApi m 10 was detected in 72.2%, 47.9%, 50.0%, 22.9%, 58.3%, and 61.8% of the patients with HBV allergy, respectively. Positive results to at least 1 HBV allergen were detected in 94.4%. IgE reactivity to Api m 3, Api m 10, or both was detected in 68.0% and represented the only HBV allergen–specific IgE in 5% of the patients. Limited inhibition of IgE binding by therapeutic HBV and limited induction of Api m 3– and Api m 10–specific IgG_4 in patients obtaining immunotherapy supports recent reports on the underrepresentation of these allergens in therapeutic HBV preparations.

Conclusion: Analysis of a panel of CCD-free HBV allergens improved diagnostic sensitivity compared with use of rApi m 1 alone, identified additional major allergens, and revealed sensitizations to allergens that have been reported to be absent or underrepresented in therapeutic HBV preparations.

(J Allergy Clin Immunol 2014;133:1383-9.)

Key words: Apis mellifera*, cross-reactive carbohydrate determinant, Hymenoptera venom, insect venom allergy, honeybee venom allergy, recombinant allergen,* Vespula vulgaris

Diagnosis of Hymenoptera venom allergy is commonly based on a history of anaphylactic sting reactions, positive skin test results, and/or detection of specific IgE to venom of honeybee or *Vespula* species.[1] Positive results on skin and serologic tests with conventional venom preparations are frequently caused by antibodies cross-reactive to conserved structures found in venom allergens. These include homologous primary structures of protein allergens (eg, hyaluronidases, dipeptidyl peptidases IV, and vitellogenins) and cross-reactive carbohydrate determinants (CCD),[2,3] which are present on the majority of Hymenoptera venom allergens.[4] Double positivity to honeybee venom (HBV) and yellow jacket venom (YJV) in patients who have not been able to identify the culprit insect necessitates additional laboratory tests (eg, IgE inhibition assays or basophil activation tests)[5,6] that are expensive, time-consuming, difficult to interpret, and therefore rarely used in the clinical routine.

Recently, the diagnostic value of IgE detection to CCD-free, species-specific recombinant Hymenoptera venom allergens, such as HBV phospholipase A_2 (rApi m 1), YJV phospholipase A_1 (rVes v 1), and antigen 5 (rVes v 5), was demonstrated.[7-14] In contrast to the situation of YJV allergy,[7,9,14,15] the frequency of sensitization to rApi m 1, the only recombinant HBV allergen commercially available to date, in patients with HBV allergy ranges from 58% to 80%,[7,8,10,13,14,16] which is insufficient to support a definitive diagnosis of HBV allergy. This suggests that additional HBV allergens are of relevance for sensitization and hence the diagnosis of HBV allergy.

The best characterized HBV allergens are phospholipase A_2 (Api m 1), hyaluronidase (Api m 2), and the basic peptide melittin (Api m 4), which all constitute medium- to high-abundance proteins.[17,18] More recently, additional HBV allergens of lower abundance have been cloned and characterized, such as acid phosphatase (Api m 3),[19] dipeptidylpeptidase IV (Api m 5),[20] Api m 6,[21] major royal jelly proteins 8 and 9 (Api m 11.0101 and Api m 11.0201),[22] icarapin (Api m 10),[23,24] and vitellogenin (Api m 12).[25] Insect cell–based expression strategies allowed for detection of IgE reactivity of these allergens independent of

From [a]the Allergy Research Group, Department of Dermatology, University Medical Center Freiburg; [b]the Institute of Biochemistry and Molecular Biology, University of Hamburg, Hamburg; [c]Thermo Fisher Scientific, Freiburg; and [d]Thermo Fisher Scientific, Uppsala.

*These authors contributed equally to this work as first authors.

‡These authors contributed equally to this work as senior authors.

Disclosure of potential conflict of interest: J. Huss-Marp is employed by and owns stock/stock options in Thermo Fisher Scientific. J. Lidholm is employed by Thermo Fisher. T. Jakob has been supported by one or more grants from, has received one or more consulting fees or honoraria from, and has received support for travel from Phadia/Thermo Fisher; has consultancy arrangements with Allergopharma, Novartis, and Jansen Cilag; has received one or more grants from or has one or more grants pending with Allergopharma, Thermo Fisher, Birken, and Cosmetic Europe; has received one or more payments for lecturing from or is on the speakers' bureau for Stallergenes, ALK-Abelló, Allergies Therapeutics, and Novartis. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication May 4, 2013; revised September 21, 2013; accepted for publication October 17, 2013.

Available online January 17, 2014.

Corresponding author: Thilo Jakob, MD, Allergy Research Group, Department of Dermatology, University Freiburg Medical Center, Hauptstrasse 7, 79104 Freiburg, Germany. E-mail: thilo.jakob@uniklinik-freiburg.de. Or: Edzard Spillner, PhD, Institute of Biochemistry and Molecular Biology, Department of Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany. E-mail: spillner@chemie.uni-hamburg.de.

0091-6749

http://dx.doi.org/10.1016/j.jaci.2013.10.060

Abbreviations used
CCD: Cross-reactive carbohydrate determinant
HBV: Honeybee venom
IQR25/75: 25% to 75% Interquartile range
sIgE: Allergen-specific IgE
YJV: Yellow jacket venom

the presence of CCDs.[12] The recombinant availability enabled analysis of different venom preparations, demonstrating that lower-abundance components, such as Api m 3 and Api m 10, although present in the crude HBV, are absent or underrepresented in preparations used for HBV immunotherapy.[23]

Here we analyzed the sensitization profile of patients with HBV allergy to a panel of CCD-free HBV allergens, including rApi m 1, rApi m 2, rApi m 3, nApi m 4, rApi m 5, and rApi m 10, by using the ImmunoCAP assay system (Thermo Fisher Scientific, Uppsala, Sweden). Inclusion of additional allergens improved the sensitivity of component-based diagnostics and demonstrated distinct sensitization profiles, some of which displayed prominent sensitizations to Api m 3 and Api m 10. In the same line, we observed a lack of Api m 3– and Api m 10–specific IgG_4 induction during HBV immunotherapy, suggesting that sensitization profiles to allergens that are not sufficiently present in therapeutic HBV preparations might be of relevance for the outcome of HBV immunotherapy.

METHODS

Patients

Sera from 184 patients with anaphylactic reactions to either honeybee (n = 144) or yellow jacket (n = 40) stings (as identified by the patient) and 40 HBV-nonallergic control subjects were analyzed. Diagnosis of HBV allergy was based on a combination of the patient's history of an anaphylactic sting reaction, a positive skin test result, and positive IgE levels to HBV (ImmunoCAP i1), as recently described.[14] As defined by the inclusion criteria, all patients with HBV allergy displayed IgE to HBV (≥0.35 kU_A/L), and 90 (62.5%) also had positive test results to YJV (ImmunoCAP i3). Diagnosis of YJV allergy was based on a combination of the patient's history of yellow jacket sting anaphylaxis, a positive skin test result, and positive IgE results for YJV (ImmunoCAP i3) and negative results for HBV (ImmunoCAP i1). The HBV-nonallergic control subjects had all experienced a bee sting, although without an anaphylactic or large local reaction. All patients and control subjects had provided informed written consent, and the study was approved by the local ethics committee.

Allergens and IgE antibody measurements

rApi m 2, rApi m 3, rApi m 5, and rApi m 10 were expressed as secreted full-length proteins by *Spodoptera frugiperda* (Sf9) insect cells, as recently described.[12,19,20,23,26,27] In brief, Sf9 cells were grown in suspension at 27°C in serum-free medium (Lonza, Verviers, Belgium) containing 10 μg/mL gentamicin (Invitrogen, Carlsbad, Calif) to a density of 1.5×10^6 cells per milliliter and then infected with a high-titer stock of recombinant baculovirus containing the allergen gene to be expressed. For protein production, the cells were incubated at 27°C and 110 rpm for 72 hours. The recombinant proteins were then purified from culture medium by using a nickel-chelating affinity matrix (NTA-agarose; Qiagen, Hilden, Germany). The purity of each recombinant protein was assessed by using SDS-PAGE (see Fig E1 in this article's Online Repository at www.jacionline.org).

Api m 4 was purified from HBV by means of sequential steps of ion exchange and size exclusion chromatography. The purity of the preparation was assessed immunologically and by using SDS-PAGE (not shown).

Experimental ImmunoCAP tests (Thermo Fisher Scientific) containing the purified HBV allergens were prepared, as previously described.[28] All IgE antibody measurements were performed with a Phadia 250 instrument, according to the manufacturer's instructions (Thermo Fischer Scientific).

Immunoreactivity of patient sera

Serum IgE reactivity was analyzed on a CAP-FEIA platform (Phadia 250) using commercially available ImmunoCAP tests for HBV (ImmunoCAP i1), YJV (ImmunoCAP i3), rApi m 1 (ImmunoCAP i208), rVes v 5 (ImmunoCAP i209), rVes v 1 (ImmunoCAP i211), and the CCD marker MUXF3 (ImmunoCAP i213) and experimental ImmunoCAP tests for rApi m 2, rApi m 3, nApi m 4, rApi m 5, and rApi m 10. Selected sera were also analyzed for IgE reactivity to major royal jelly protein 8 and 9 (Api m 11.0101 and Api m 11.0201) and 3 additional HBV proteins (not been assigned as allergens) by using ELISA, as recently described.[22] Allergen-specific IgG_4 reactivity to rApi m 1, nApi m 4, rApi m 3, and rApi m 10 in selected sera was analyzed by using a Phadia 250 instrument and 1:100 or 1:20 serum dilutions.

CAP-FEIA inhibition

Inhibition of allergen-specific IgE (sIgE) binding to HBV (ImmunoCAP i1) by nApi m 1 (Latoxan, Valence, France), rApi m 3, or rApi m 10 was performed by means of preincubation of patient sera and inhibitors at the indicated concentrations for 2 hours at room temperature before the CAP-FEIA analysis. Alternatively, sera were preincubated with a crude HBV preparation (Latoxan) or solubilized freeze-dried therapeutic HBV preparations (ie, not absorbed to alum) at 300 μg/mL.

RESULTS

IgE reactivity to HBV allergens in patients with HBV allergy, patients with YJV allergy, and HBV-nonallergic control subjects

IgE reactivity (≥0.35 kU_A/L) to the commercially available rApi m 1 (i208) was detected in 72.2%, to rApi m 2 in 47.9%, to rApi m 3 in 50.0%, to nApi m 4 in 22.9%, to rApi m 5 in 58.3%, and to rApi m 10 in 61.8% of patients with HBV allergy (Fig 1). In patients with YJV allergy, no relevant IgE reactivity was detected, except to rApi m 5 (3/40, Fig 1), the cross-reactive dipeptidylpeptidase also present in YJV as Ves v 3. Of the 40 HBV-nonallergic control subjects, 6 (15%) displayed IgE reactivity of 0.35 kU_A/L or greater to HBV (ImmunoCAP i1), which is in line with previous reports.[29] In this subgroup of 6 control subjects, IgE reactivity to rApi m 1 was detected in 3, to rApi m 5 in 2, and to rApi m 10 in 1 subjects. No IgE reactivity to any of the tested HBV allergens was detected in the ImmunoCAP i1 negative control sera (Fig 1). Among the patients with HBV allergy, positive results to at least 1 HBV allergen were detected in 94.4%, and positive results to at least 1 of the HBV-specific allergens Api m 1, 3, 4, or 10 were detected in 89.6% (Fig 2). The majority of patients with HBV allergy were sensitized to more than 1 allergen (74.3%), and a minority (9.7%) were sensitized to all allergens tested. Interestingly, HBV-monosensitized patients (n = 54) had lower total IgE levels, lower levels of sIgE to HBV (ImmunoCAP i1), and lower levels of sIgE to all HBV allergens tested when compared with patients with HBV allergy who were also sensitized to YJV (ImmunoCAP i3, n = 90; see Table E1 in this article's Online Repository at www.jacionline.org).

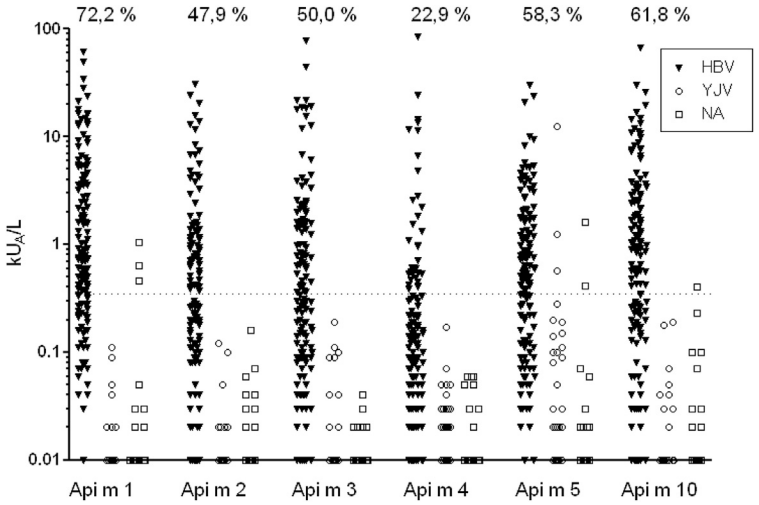

FIG 1. IgE immunoreactivity of individual patient sera with recombinant allergens. IgE reactivity to HBV allergens of sera from patients with HBV allergy (n = 144), patients with YJV allergy (n = 40), and HBV-exposed but nonallergic control subjects (*NA*; n = 40). The lower-end cutoff of the CAP-FEIA (<0.35 kU$_A$/L) is represented as a *dotted line*.

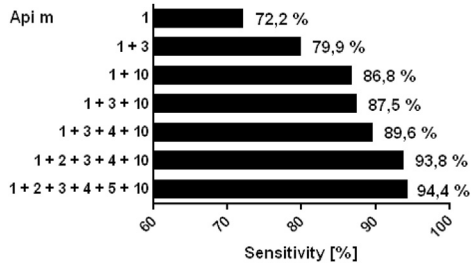

FIG 2. Diagnostic sensitivity of sIgE to different combinations of HBV allergens. Detection of IgE reactivity to a panel of HBV allergens increases diagnostic sensitivity in patients with HBV allergy (n = 144).

Sensitization profiles in patients with HBV allergy

Among the patients with HBV allergy, 39 of 64 possible different sensitization profiles were present, and the 10 most frequent profiles covered 64% of the study population (see Table E3 in this article's Online Repository at www.jacionline.org). As suggested from analysis of IgE profile complexity,[30] the number of allergens detected showed a clear association with the concentration of sIgE to HBV. Interestingly, the HBV monosensitized patients mostly display lower sIgE levels to lower numbers of allergens (cluster on the left side), while the HBV and YJV double-sensitized patients recognize multiple bee venom allergens (cluster on the right; see Fig E2 in this article's Online Repository at www.jacionline.org). IgE reactivity to Api m 3, Api m 10, or both was detected in 68% of the patients, and 7 (4.8%) patients displayed IgE reactivity exclusively to Api m 3, Api m 10, or both. This is of particular interest because Api m 3 and Api m 10 have been demonstrated to be absent or underrepresented in HBV preparations used for immunotherapy.[19,23]

IgE reactivity to HBV allergens in relation to whole HBV

IgE reactivity to HBV (ImmunoCAP i1) displayed a significant correlation ($r = 0.94$, $P < .0001$) with the sum of IgE reactivity to Api m 1, Api m 2, Api m 3, Api m 4, Api m 5, Api m 10, and CCDs (Fig 3, *A*). The relative contribution of sIgE to the different allergens was calculated in relation to and expressed as a percentage of sIgE to HBV (ImmunoCAP i1; Fig 3, *B* and *C*). The relative IgE reactivity to Api m 3 (median, 7%; 25% to 75% interquartile range [IQR25/75], 3%/14%) and Api m 10 (median, 14%; IQR25/75, 5%/28%), even though lower than the relative IgE reactivity to Api m 1 (median, 20%; IQR25/75, 9%/49%), suggests a relevant role in HBV allergy.

CAP-FEIA inhibition experiments with titrated doses of recombinant allergens in equimolar concentrations (Fig 4, *A*) in patients either sensitized to Api m 1 and not to Api m 10 (Api m 1^+ Api m 10^-) or *vice versa* (Api m 1^- Api m 10^+) confirmed the relative contribution of IgE directed against Api m 1 and Api m 10. Similarly, the degree of maximal inhibition with Api m 1, Api m 3, and Api m 10 correlated with the calculated relative IgE reactivity (Fig 4, *B*). Inhibition of HBV sIgE reactivity by different HBV preparations, such as crude HBV or therapeutic preparations, provided a means to demonstrate the presence of individual allergens in the preparation. For the predominantly Api m 1–positive sera, both a crude and a therapeutic HBV preparation blocked the IgE binding to a similar degree. In contrast, in predominantly Api m 10–positive sera (relative IgE reactivity, 54%; range, 35% to 72%), therapeutic HBV preparations were clearly less effective compared with a crude HBV preparation (Fig 4, *C*). This result is consistent with the previously reported absence of Api m 10 from therapeutic HBV preparations.[23]

HBV allergen–specific IgG$_4$ during HBV immunotherapy

Finally, we analyzed IgG$_4$ responses to the HBV-specific allergens Api m 1, Api m 3, Api m 4, and Api m 10 in 20 patients who had undergone HBV immunotherapy for 12 to 48 months. A prominent induction of sIgG$_4$ was observed for the 2 highly abundant allergens Api m 1 and Api m 4, which was comparable with that observed with whole HBV. In contrast, no or very little sIgG$_4$ induction was observed for the low-abundance allergens

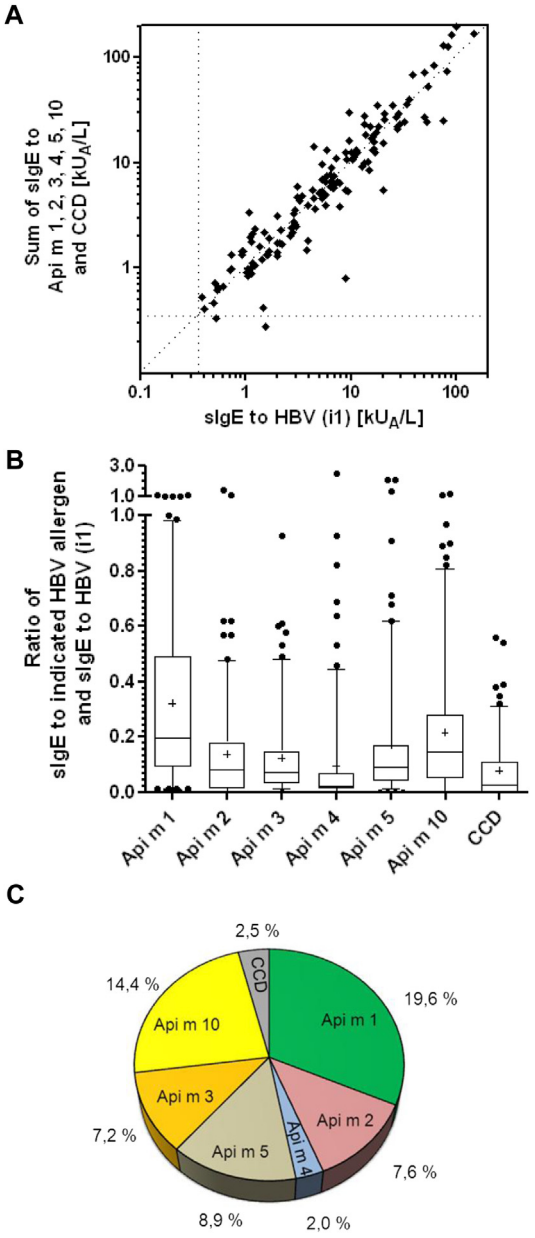

FIG 3. IgE reactivity to single HBV allergens in relation to sIgE to HBV. **A,** IgE reactivity to HBV (ImmunoCAP i1) in patients with HBV allergy (n = 144) in relation to the sum of IgE reactivity to Api m 1, Api m 2, Api m 3, Api m 4, Api m 5, Api m 10, and CCDs. **B,** The relative sIgE reactivity to single HBV allergens was calculated as a ratio of sIgE reactivity to HBV and displayed as a whisker plot with medians; 5th, 25th, 75th, and 95th percentiles; and outliers. **C,** The median was used to present the relative contribution to IgE reactivity of single allergens as a pie chart.

Api m 3 and Api m 10 (Fig 5), again supporting the notion that Api m 3 and Api m 10 might be underrepresented in therapeutic HBV preparations.

DISCUSSION

In this study we addressed the component resolution of IgE sensitization in a large set of sera from patients with HBV allergy to a broad panel of different recombinant high- and low-abundance HBV allergens. Component resolution at this level in clinical diagnosis of HBV allergy is not yet possible because of the commercial unavailability of most of the HBV allergens required. Recently, we and others have extended the small set of allergens in the databases, including Api m 1, Api m 2, and Api m 4, to a broad panel of allergens up to Api m 12.[25]

For the production of complex and often high-molecular-weight Hymenoptera venom allergens, insect cells recently turned out to be an appropriate system in terms of functionality, epitope authenticity, glycosylation, and folding.[12,20,31] IgE with specificity for CCDs plays a key role in allergen cross-reactivity and represents a major concern for the specificity of diagnostic approaches in patients with Hymenoptera venom allergy.[2,4,5,12] We recently demonstrated that the use of Sf9 insect cells for allergen expression represents a strategy to circumvent the establishment of CCDs while maintaining the advantages of a nearly autologous expression system.[12,22,23,26] A recent study also suggested that the IgE reactivity of rApi m 1 compared with the native protein is not affected by the presence of a his-tag.[16] In addition, using his-tagged recombinant maltose binding protein, we did not observe any his-tag–based IgE reactivity in patients with HBV allergy (data not shown).

Almost all patients with HBV allergy in this study (136/144) proved to have specific IgE antibodies against at least 1 of the HBV allergens included in our panel. Api m 1 remains the most frequently recognized allergen, and its introduction in recombinant and CCD-free forms has been helpful toward improved clinical diagnosis and a better understanding of the molecular sensitization pattern in HBV allergy.[7,16] Although it is clearly justified to regard Api m 1, through frequent sensitization and high abundance in HBV, as the single most important determinant of HBV allergenicity, the results presented here demonstrate that sensitization to HBV is considerably more complex than previously recognized.

In the present study nearly half of the patients with HBV allergy displayed IgE reactivity against the HBV hyaluronidase Api m 2, supporting the previously reported role of Api m 2 as the relevant HBV allergen.[12,14] Double positivity to HBV and YJV, apart from CCDs, in patients with venom allergy has previously been largely attributed to IgE directed against either hyaluronidases (Api m 2 and Ves v 2)[32] or dipeptidylpeptidases (Api m 5 and Ves v 3).[20] However, recent studies have indicated that cross-reactivity between hyaluronidases is limited.[12,33,34] The acid phosphatase Api m 3 is a classical and species-specific allergen without homologues in YJV that has been cloned recently.[19] IgE reactivity to rApi m 3 in 50% of the patients with HBV allergy corroborates its relevance as a major HBV allergen.

The peptidic HBV allergen Api m 4 is the only nonrecombinant component used in this study. Even though it represents the bulk of the venom dry weight and 2 patients showed detectable IgE exclusively to Api m 4 (0.54 and 0.40 kU_A/L), the moderate frequency of sensitization and its low overall contribution to IgE binding to whole HBV suggests a limited clinical importance.

IgE reactivity to the dipeptidyl peptidase IV allergen Api m 5 in 58% and to Api m 10 in 62% of the patient population establishes both as major allergens in HBV allergy. Similar to Api m 1, Api m 3, and Api m 4, Api m 10 is a species-specific allergen and hence constitutes an important molecule for diagnostic and therapeutic considerations.

It is evident from our data that Api m 1, Api m 3, Api m 5, and Api m 10 are major HBV allergens. This number is higher than anticipated, and inclusion of additional major allergens into

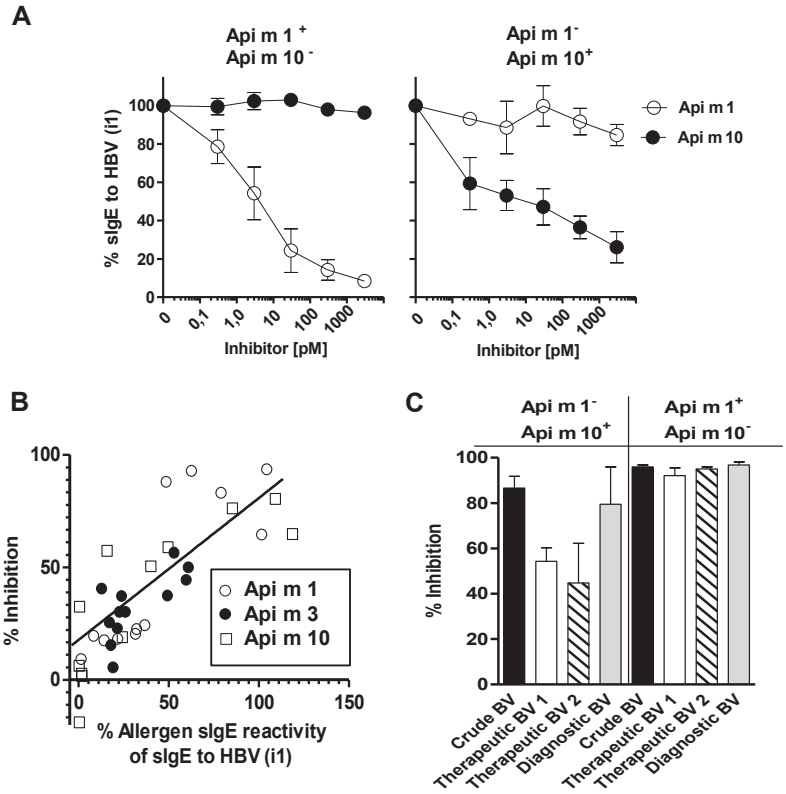

FIG 4. Inhibition of IgE reactivity to HBV (ImmunoCAP i1) by single HBV allergens or crude or therapeutic HBV preparations. **A,** CAP-FEIA inhibition of sIgE reactivity to HBV (ImmunoCAP i1) was performed with Api m 1 or Api m 10 as inhibitors at increasing concentrations in patients with predominant Api m 1 sensitization (Api m 1^+ Api m 10^-; n = 3, mean ± SD) or in patients with a predominant Api m 10 sensitization (Api m 1^- Api m 10^+; n = 3, mean ± SD). **B,** CAP-FEIA inhibition of sIgE reactivity to HBV (ImmunoCAP i1) was performed with Api m 1, Api m 3, or Api m 10 at 300 nmol/L in patients with HBV allergy (n = 36). The degree of CAP-FEIA inhibition was correlated with the relative IgE reactivity to Api m 1, Api m 3, or Api m 10, as calculated in Fig 3 ($P < .0001$, $r = .8082$). **C,** CAP-FEIA inhibition of sIgE reactivity to HBV (ImmunoCAP i1) was performed with a crude HBV, a diagnostic HBV, or 2 different therapeutic HBV preparations at 300 μg/mL in patients with predominant Api m 1 sensitization (Api m 1^+ Api m 10^-; n = 4, mean ± SD) or in patients with predominant Api m 10 sensitization (Api m 1^- Api m 10^+; n = 4, mean ± SD). *BV*, Bee venom.

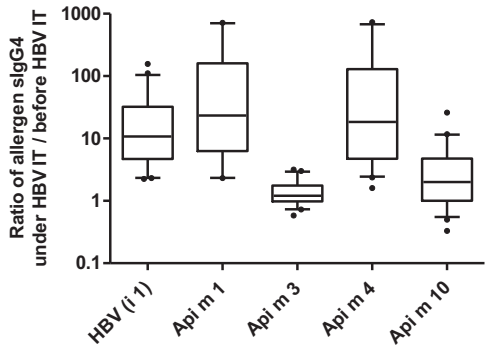

FIG 5. Induction of allergen-specific IgG_4 during HBV immunotherapy. $sIgG_4$ responses to Api m 1, Api m 3, Api m 4, and Api m 10 were analyzed in patients before and 12 to 36 months after initiation of HBV immunotherapy (n = 20). The induction of $sIgG_4$ was expressed as the ratio of $sIgG_4$ during immunotherapy/$sIgG_4$ before immunotherapy and is displayed as whisker plots with medians; 5th, 25th, 75th, and 95th percentiles; and outliers.

diagnostic serology would likely contribute to improved clinical diagnosis. In our opinion the best approach to apply these new components would be to add them to the repertoire of available allergens, either as single components or as selected combinations of recombinant allergens that allow species-specific diagnosis of HBV allergy in those patients who display HBV and YJV double-positive results and thus cannot receive clear diagnosis by using extract-based tests.

Among the patients with HBV allergy who displayed sIgE to HBV (ImmunoCAP i1) but had negative results for Api m 1 (n = 40), IgE reactivity was detected in 47.5% to Api m 2, in 27.5% to Api m 3, in 17.5% to Api m 4, in 40% to Api m 5, and in 52.5% to Api m 10. Sensitization to only 1 allergen was observed in 29 patients (Api m 1, n = 17; Api m 2, n = 3; Api m 4, n = 2; Api m 5, n = 1; and Api m 10, n = 6). Thus all allergens included here demonstrated a potential additional value in the molecular diagnostics of HBV allergy. In contrast, sera from patients with a convincing history of anaphylactic bee sting reactions that were negative for sIgE to HBV (ImmunoCAP i1, n = 14) and thus not included in our study population were also negative for sIgE to all of the HBV-specific components tested (see Table E4 in this article's Online Repository at www.jacionline.org).

Because vespid homologues exist for both Api m 2 and Api m 5, we expected some of the IgE reactivity to these allergens to be related to a concomitant sensitization to YJV, which is in contrast

to the HBV-specific allergens Api m 1, Api m 3, Api m 4, and Api m 10. Interestingly, in patients with HBV allergy, concomitant sensitization to YJV was associated with a higher level of total IgE and HBV sIgE (ImmunoCAP i1), as well as higher levels of sIgE to all HBV allergens tested (see Table E1), suggesting effects that were independent of cross-reactivity at the protein level. We observed the same phenomenon (higher total and sIgE levels in double-sensitized compared with monosensitized patients) in a separate population of patients with YJV allergy (n = 170, see Table E2 in this article's Online Repository at www.jacionline.org),[15] suggesting that this might reflect a more advanced state of atopic immune deviation in the double-sensitized population compared with the monosensitized population. This is in part supported by the observation that HBV-monosensitized patients mostly display lower sIgE levels to lower numbers of allergens, whereas double-sensitized patients recognize multiple bee venom allergens (see Fig E2 in this article's Online Repository at www.jacionline.org). Similar findings have recently also been reported for sensitization to *Phleum pratense* allergens.[35]

A few sera of patients with HBV allergy who displayed sIgE to HBV (ImmunoCAP i1) were found to be negative for all HBV-specific allergens. These sera were additionally tested by using additional HBV proteins, including Api m 11.0101 and Api m 11.0201[22] and 3 novel components (a venom protease, C1q, and PVF-1, which have not been designated as allergens thus far; data not shown). Thereby a further 3 sera were found to be positive: 1 for the venom protease, 1 for Api m 11.0201 and C1q, and 1 for C1q. These results clearly suggest that increasing the numbers of components certainly can increase the sensitivity of component-resolved diagnostics to a level at which virtually all patients with HBV allergy can be detected. These data also show that the individual sensitization profiles of patients with HBV allergy are more complex than anticipated. The level of complexity of patients' sensitization patterns clearly correlates with HBV-specific IgE levels, a finding that is similar to those reported from component-resolved studies in pollen-sensitized patients.[30,36] Notably, 39 of 64 possible different sensitization profiles were present, and the 10 most frequent profiles covered 64% of our study population (see Table E3). In an attempt to estimate the contribution of Api m 10 compared with Api m 1, we calculated the relative IgE reactivity in relation to IgE to HBV (ImmunoCAP i1). In the entire population with HBV allergy, IgE reactivity to rApi m 10 was on the order of two thirds of that to rApi m 1. Because this approach allows an approximation only, we performed CAP inhibition with Api m 1, Api m 3, or Api m 10 in patients who showed a predominant sensitization to either of the allergens. The degree of maximal inhibition with Api m 1, Api m 3, and Api m 10 correlated well with the calculated relative IgE reactivity, suggesting that at least for these 3 allergens, this parameter can be used to estimate the magnitude of IgE binding of the respective allergen.

In light of the prominent IgE reactivity to Api m 10 and the recent report of absence or underrepresentation of low-abundance allergens, such as Api m 10 and Api m 3, in therapeutic HBV preparations, we analyzed the efficacy of different HBV preparations to block IgE binding to HBV (ImmunoCAP i1) in patients who were predominantly sensitized to Api m 10. Our inhibition studies clearly suggested that Api m 10 is underrepresented in the therapeutic HBV preparations when compared with the crude HBV. If a patient's IgE reactivity to HBV (ImmunoCAP i1) predominantly comprises IgE to Api m 10, the use of therapeutic HBV preparations apparently lacking Api m 10 might not lead to the desired therapeutic tolerance induction. With the tools of component-resolved diagnostics at our hands, we might be able to address this issue.

As a first step in this direction, we simply addressed the question of whether HBV immunotherapy leads to specific IgG_4 induction to the respective HBV allergens. In contrast to the prominent induction of $sIgG_4$ against the 2 high-abundance allergens Api m 1 and Api m 4, no or very little induction of $sIgG_4$ to Api 3 and Api m 10 was observed. This observation is consistent with previous reports that Api m 3 and Api m 10 are underrepresented in therapeutic HBV preparations.[9,23]

In summary, the analysis of IgE reactivity to a large panel of CCD-free bee venom allergens improves the sensitivity and precision of component-based diagnostics in patients with HBV allergy. In addition, the component resolution allowed the identification of distinct sensitization profiles. Prominent IgE reactivity to some allergens that are absent or underrepresented in therapeutic HBV preparations suggests that different profiles might be of relevance for the success of HBV immunotherapy. Future studies will need to address these issues, in particular whether distinct HBV sensitization profiles can be used as predictors for the outcome of HBV immunotherapy.

We are grateful for the expert technical assistance of Britta Dorn, Andrea Komann, Heike Neuman, and Frauke Planz (Freiburg, Germany). Dr Lisa Elfström and Ms Maria Ahlberg (Uppsala, Sweden) are gratefully acknowledged for the purification of nApi m 4 (L.E.) and the preparation of experimental ImmunoCAP tests (L.E. and M.A.).

Clinical implications: Patients with bee venom allergy display distinct sensitization profiles to a panel of HBV allergens, some of which have been reported to be absent or underrepresented in therapeutic HBV preparations.

REFERENCES

1. Bilo BM, Rueff F, Mosbech H, Bonifazi F, Oude-Elberink JN. Diagnosis of Hymenoptera venom allergy. Allergy 2005;60:1339-49.
2. Aalberse RC, Akkerdaas J, van Ree R. Cross-reactivity of IgE antibodies to allergens. Allergy 2001;56:478-90.
3. Tretter V, Altmann F, Kubelka V, Marz L, Becker WM. Fucose alpha 1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. Int Arch Allergy Immunol 1993;102:259-66.
4. Hemmer W, Focke M, Kolarich D, Dalik I, Gotz M, Jarisch R. Identification by immunoblot of venom glycoproteins displaying immunoglobulin E-binding N-glycans as cross-reactive allergens in honeybee and yellow jacket venom. Clin Exp Allergy 2004;34:460-9.
5. Jappe U, Raulf-Heimsoth M, Hoffmann M, Burow G, Hubsch-Muller C, Enk A. In vitro hymenoptera venom allergy diagnosis: improved by screening for cross-reactive carbohydrate determinants and reciprocal inhibition. Allergy 2006;61:1220-9.
6. Eberlein B, Krischan L, Darsow U, Ollert M, Ring J. Double positivity to bee and wasp venom: improved diagnostic procedure by recombinant allergen-based IgE testing and basophil activation test including data about cross-reactive carbohydrate determinants. J Allergy Clin Immunol 2012;130:155-61.
7. Hofmann SC, Pfender N, Weckesser S, Huss-Marp J, Jakob T. Added value of IgE detection to rApi m 1 and rVes v 5 in patients with Hymenoptera venom allergy. J Allergy Clin Immunol 2011;127:265-7.
8. Korosec P, Valenta R, Mittermann I, Celesnik N, Erzen R, Zidarn M, et al. Low sensitivity of commercially available rApi m 1 for diagnosis of honeybee venom allergy. J Allergy Clin Immunol 2011;128:671-3.
9. Korosec P, Valenta R, Mittermann I, Celesnik N, Silar M, Zidarn M, et al. High sensitivity of CAP-FEIA rVes v 5 and rVes v 1 for diagnosis of *Vespula* venom allergy. J Allergy Clin Immunol 2012;129:1406-8.

10. Mittermann I, Zidarn M, Silar M, Markovic-Housley Z, Aberer W, Korosec P, et al. Recombinant allergen-based IgE testing to distinguish bee and wasp allergy. J Allergy Clin Immunol 2010;125:1300-7.e3.
11. Muller UR. Bee venom allergy in beekeepers and their family members. Curr Opin Allergy Clin Immunol 2005;5:343-7.
12. Seismann H, Blank S, Braren I, Greunke K, Cifuentes L, Grunwald T, et al. Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation. Mol Immunol 2010;47:799-808.
13. Sturm GJ, Hemmer W, Hawranek T, Lang R, Ollert M, Spillner E, et al. Detection of IgE to recombinant Api m 1 and rVes v 5 is valuable but not sufficient to distinguish bee from wasp venom allergy. J Allergy Clin Immunol 2011;128: 247-8.
14. Hofmann SC, Pfender N, Weckesser S, Blank S, Huss-Marp J, Spillner E, et al. Detection of IgE to recombinant Api m 1 and rVes v 5 is valuable but not sufficient to distinguish bee from wasp venom allergy. Reply. J Allergy Clin Immunol 2011; 128:248.
15. Vos B, Kohler J, Muller S, Stretz E, Rueff F, Jakob T. Spiking venom with rVes v 5 improves sensitivity of IgE detection in patients with allergy to *Vespula* venom. J Allergy Clin Immunol 2013;131:1225-7.e1.
16. Jakob T, Kohler J, Blank S, Magnusson U, Huss-Marp J, Spillner E, et al. Comparable IgE reactivity to natural and recombinant Api m 1 in cross-reactive carbohydrate determinant-negative patients with bee venom allergy. J Allergy Clin Immunol 2012;130:276-8.
17. Arbesman CE, Reisman RE, Wypych JI. Allergenic potency of bee antigens measured by RAST inhibition. Clin Allergy 1976;6:587-95.
18. Müller UR. Insektenstichallergie: Klinik, Diagnostik und Therapie. Stuttgart: Gustav Fischer Verlag; 1988.
19. Grunwald T, Bockisch B, Spillner E, Ring J, Bredehorst R, Ollert MW. Molecular cloning and expression in insect cells of honeybee venom allergen acid phosphatase (Api m 3). J Allergy Clin Immunol 2006;117:848-54.
20. Blank S, Seismann H, Bockisch B, Braren I, Cifuentes L, McIntyre M, et al. Identification, recombinant expression, and characterization of the 100 kDa high molecular weight Hymenoptera venom allergens Api m 5 and Ves v 3. J Immunol 2010;184:5403-13.
21. Michel Y, McIntyre M, Ginglinger H, Ollert M, Cifuentes L, Blank S, et al. The putative serine protease inhibitor Api m 6 from *Apis mellifera* venom: recombinant and structural evaluation. J Investig Allergol Clin Immunol 2012; 22:476-84.
22. Blank S, Bantleon FI, McIntyre M, Ollert M, Spillner E. The major royal jelly proteins 8 and 9 (Api m 11) are glycosylated components of *Apis mellifera* venom with allergenic potential beyond carbohydrate-based reactivity. Clin Exp Allergy 2012;42:976-85.
23. Blank S, Seismann H, Michel Y, McIntyre M, Cifuentes L, Braren I, et al. Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts. Allergy 2011;66:1322-9.
24. Peiren N, de Graaf DC, Brunain M, Bridts CH, Ebo DG, Stevens WJ, et al. Molecular cloning and expression of icarapin, a novel IgE-binding bee venom protein. FEBS Lett 2006;580:4895-9.
25. Blank S, Seismann H, McIntyre M, Ollert M, Wolf S, Bantleon FI, et al. Vitellogenins are new high molecular weight components and allergens (Api m 12 and Ves v 6) of *Apis mellifera* and *Vespula vulgaris* venom. PLoS One 2013; 8:e62009.
26. Blank S, Michel Y, Seismann H, Plum M, Greunke K, Grunwald T, et al. Evaluation of different glycoforms of honeybee venom major allergen phospholipase A2 (Api m 1) produced in insect cells. Protein Pept Lett 2011;18: 415-22.
27. Seismann H, Blank S, Cifuentes L, Braren I, Bredehorst R, Grunwald T, et al. Recombinant phospholipase A1 (Ves v. 1) from yellow jacket venom for improved diagnosis of hymenoptera venom hypersensitivity. Clin Mol Allergy 2010;8:7.
28. Marknell DeWitt A, Niederberger V, Lehtonen P, Spitzauer S, Sperr WR, Valent P, et al. Molecular and immunological characterization of a novel timothy grass *(Phleum pratense)* pollen allergen, Phl p 11. Clin Exp Allergy 2002;32: 1329-40.
29. Bilo BM, Bonifazi F. Epidemiology of insect-venom anaphylaxis. Curr Opin Allergy Clin Immunol 2008;8:330-7.
30. Tripodi S, Frediani T, Lucarelli S, Macri F, Pingitore G, Di Rienzo Businco A, et al. Molecular profiles of IgE to *Phleum pratense* in children with grass pollen allergy: implications for specific immunotherapy. J Allergy Clin Immunol 2012; 129:834-9.e8.
31. Soldatova LN, Crameri R, Gmachl M, Kemeny DM, Schmidt M, Weber M, et al. Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with *Escherichia coli*. J Allergy Clin Immunol 1998;101:691-8.
32. King TP, Joslyn A, Kochoumian L. Antigenic cross-reactivity of venom proteins from hornets, wasps, and yellow jackets. J Allergy Clin Immunol 1985; 75:621-8.
33. Jin C, Focke M, Leonard R, Jarisch R, Altmann F, Hemmer W. Reassessing the role of hyaluronidase in yellow jacket venom allergy. J Allergy Clin Immunol 2010; 125:184-90.e1.
34. Skov LK, Seppala U, Coen JJ, Crickmore N, King TP, Monsalve R, et al. Structure of recombinant Ves v 2 at 2.0 Angstrom resolution: structural analysis of an allergenic hyaluronidase from wasp venom. Acta Crystallogr D Biol Crystallogr 2006;62:595-604.
35. Hatzler L, Panetta V, Lau S, Wagner P, Bergmann RL, Illi S, et al. Molecular spreading and predictive value of preclinical IgE response to *Phleum pratense* in children with hay fever. J Allergy Clin Immunol 2012;130:894-901.e5.
36. Moverare R, Petays T, Vartiainen E, Haahtela T. IgE reactivity pattern to timothy and birch pollen allergens in Finnish and Russian Karelia. Int Arch Allergy Immunol 2005;136:33-8.

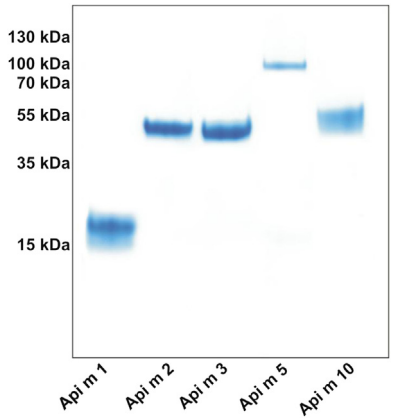

FIG E1. Recombinant expression and immunoreactivity of venom allergens. SDS-PAGE analyses of the purified recombinant allergens Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10, as visualized by using Coomassie blue staining.

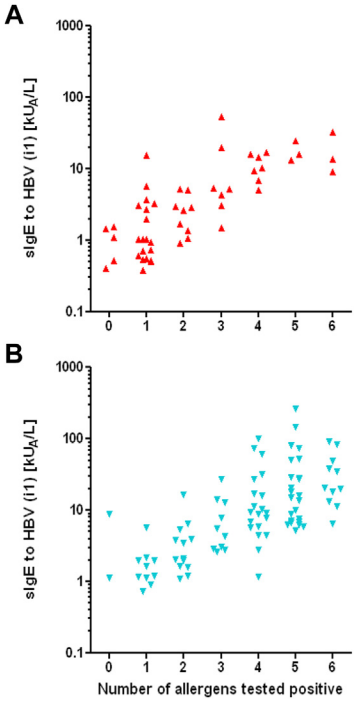

FIG E2. Relationship between levels of sIgE to HBV and the number of different HBV allergens recognized in HBV-monosensitized **(A)** and HBV and YJV double-sensitized patients **(B)**.

TABLE E1. Total IgE, sIgE to BV extract and to CCD-free HBV allergens Api m 1, Api m 2, Api m 3, Api m 4, Api m 5 and Api m 10 in HBV-allergic patients monosensitized to HBV extract or double sensitized to HBV and YJV extract

	Monosensitized to HBV (n = 54)			Double sensitized to HBV + YJV (n = 90)		
Total IgE (kU/L)	40.9 (31.5-53.0)	[4.20-260]		139 (111-175)	[13.8-1374]***	
sIgE to HBV (i1) (kU_A/L)	2.96 (2.08-4.22)	[0.38-53.8]		8.55 (6.49-11.3)	[0.73-268]***	
sIgE to rApi m 1 (kU_A/L)	0.67 (0.41-1.09)	[0.01-49.7]	67%	1.28 (0.89-1.86)	[0.06-108]*	76%
sIgE to rApi m 2 (kU_A/L)	0.08 (0.05-0.14)	[0.00-13.0]	28%	0.59 (0.40-0.87)	[0.00-113]***	60%
sIgE to rApi m 3 (kU_A/L)	0.15 (0.09-0.24)	[0.01-12.6]	28%	0.72 (0.50-1.04)	[0.03-77.8]***	63%
sIgE to nApi m 4 (kU_A/L)	0.08 (0.05-0.13)	[0.00-24.2]	17%	0.18 (0.13-0.26)	[0.00-84.8]***	27%
sIgE to rApi m 5 (kU_A/L)	0.26 (0.16-0.41)	[0.01-8.27]	39%	0.64 (0.45-0.89)	[0.02-30.2]**	70%
sIgE to Api m 10 (kU_A/L)	0.24 (0.14-0.41)	[0.00-14.4]	43%	1.08 (0.73-1.59)	[0.00-66.6]***	73%

Monosensitized, ie, HBV-allergic patients sensitized to BV extract (i1) only (n = 54); double sensitized, ie, HBV-allergic patients sensitized to both HBV extract (i1) and YJV extract (i3) (n = 90). Geometric mean (95% CI) [Range]; %; percentage of patients having sIgE ≥0.35 kU_A/L; *$P < .05$; **$P < .01$; ***$P < .001$ (Mann-Whitney U test).

TABLE E2. Total IgE, sIgE to YJV extract and to CCD-free YJV allergens Ves v 1 and Ves v 5 in YJV allergic patients monosensitized to YJV extract or double sensitized to YJV and BV extract

	Monosensitized to YJV (n = 103)			Double sensitized to YJV + HBV (n = 67)		
Total IgE (kU/L)	44.8 (36.9-54.3)	[3.50-492]		134 (100-180)	[5.9-1589]***	
sIgE to YJV (i3) (kU_A/L)	2.73 (2.14-4.49)	[0.42-34.0]		8.28 (6.35-10.8)	[0.56-54.7]***	
sIgE to rVes v 1 (kU_A/L)	0.16 (0.10-0.25)	[0.00-42.7]	39%	0.73 (0.41-1.29)	[0.00-67.0]***	66%
sIgE to rVes v 5 (kU_A/L)	1.83 (1.35-2.47)	[0.01-40.1]	92%	4.06 (2.63-6.27)	[0.01-56.1]***	94%

To address if the phenomenon (higher sIgE in double sensitized as compared to monosensitized patients) is specific for HBV allergy (Table E1) or a more general phenomenon we analyzed a population of YJV allergic patients (n = 170). The diagnosis of YJV allergy was based on a positive anaphylactic sting reaction to YJ (as identified by the patient), a positive skin test and positive sIgE to YJV extract i3 as recently described.[15] When comparing monosensitized to double-sensitized patients we observed the same pattern, ie, higher total IgE and higher specific IgE to YJV or to the YJV allergens rVes v 5 and rVes v 1. Since both allergens are CCD-free and species-specifc (ie, have no corresponding homologous allergen in HBV) this data confirms our initial interpretation that this observation is independent of CCD or protein cross-reactivity. Monosensitized, ie, YJV-allergic patients sensitized to YJV extract (i3) only (n = 103); double sensitized, ie, YJV-allergic patients sensitized to both YJV extract (i3) and BV extract (i1) (n = 67). Geometric mean (95 % CI) [Range]; %; percentage of patients having sIgE ≥0.35 kU_A/L; ***$P < .001$ (Mann-Whitney *U* test).

TABLE E3. Sensitization profiles to 6 HBV allergens in 144 patients with HBV allergy ordered in decreasing frequency

Api m 1	Api m 2	Api m 3	Api m 4	Api m 5	Api m 10	No.	Percent	Cumulative percent
•	•	•		•	•	21	14.58	14.58
•						17	11.81	26.39
•	•	•	•	•	•	14	9.72	36.11
•		•		•	•	11	7.64	43.75
	•	•		•	•	6	4.17	47.92
					•	6	4.17	52.08
•					•	5	3.47	55.56
•	•			•		4	2.78	58.33
•		•	•	•	•	4	2.78	61.11
•				•		4	2.78	63.89
•		•		•		3	2.08	65.97
	•			•		3	2.08	68.06
	•				•	3	2.08	70.14
	•					3	2.08	72.22
•	•	•		•		2	1.39	73.61
•	•		•		•	2	1.39	75.00
•	•				•	2	1.39	76.39
•		•			•	2	1.39	77.78
•		•				2	1.39	79.17
•			•		•	2	1.39	80.56
•				•	•	2	1.39	81.94
			•			2	1.39	83.33
•	•	•			•	1	0.69	84.03
•	•		•	•	•	1	0.69	84.72
•	•		•			1	0.69	85.42
•	•			•	•	1	0.69	86.11
•	•					1	0.69	86.81
•		•	•	•		1	0.69	87.50
•			•			1	0.69	88.19
	•	•	•	•	•	1	0.69	88.89
	•	•	•	•		1	0.69	89.58
	•	•			•	1	0.69	90.28
	•		•	•	•	1	0.69	90.97
		•	•	•	•	1	0.69	91.67
		•			•	1	0.69	92.36
			•	•		1	0.69	93.06
				•	•	1	0.69	93.75
				•		1	0.69	94.44
						8	5.56	100.00
						144	100.00	

Dots indicate the presence of sIgE ($\geq$0.35 kU_A/L).

TABLE E4. sIgE profile to recombinant HBV allergens of patients with a convincing history of anaphylactic bee sting reactions that were negative (<0.35 KU/L) for sIgE to HBV extract (n = 14)

	Clinical sting reaction	tIgE	sIgE to HBV (ImmunoCAP i1)	sIgE to Api m 1	sIgE to Api m 3	sIgE to Api m 4	sIgE to Api m 10	Skin test	
Patient no.	Anaphylaxis grade (Ring and Messmer)	kU/L	kU_A/L	kU_A/L	kU_A/L	kU_A/L	kU_A/L	HBV SPT	HBV i.c.
1	2	28.5	0.21	0.06	0.00	0.00	0.08	+	ND
2	3	<2.0	0.09	0.05	0.02	0.02	0.02	−	+
3	1	34.80	0.06	0.00	0.00	0.00	0.00	+	ND
4	1	74.70	0.08	0.02	0.01	0.02	0.01	−	−
5	3	74.60	0.08	0.03	0.01	0.03	0.02	−	−
6	2	57.50	0.12	0.09	0.06	0.08	0.08	−	−
7	3	29.00	0.31	0.00	0.05	0.00	0.10	−	−
8	2	531.00	0.05	0.00	0.00	0.00	0.00	−	−
9	2	42.10	0.06	0.00	0.00	0.01	0.01	−	−
10	1	32.20	0.10	0.05	0.03	0.05	0.04	+	ND
11	3	12.60	0.05	0.00	0.00	0.00	0.00	−	−
12	1	231.00	0.34	0.19	0.07	0.00	0.03	−	−
13	3	23.90	0.27	0.05	0.02	0.00	0.01	−	+
14	2	41.50	0.25	0.01	0.00	0.02	0.02	−	−

tIgE, Total Ig; *i.c.*, intracutaneous.