

Original article

Hymenoptera venom allergy: analysis of double positivity to honey bee and *Vespula* venom by estimation of IgE antibodies to species-specific major allergens Api m1 and Ves v5

Background: In patients with hymenoptera venom allergy diagnostic tests are often positive with honey bee and *Vespula* venom causing problems in selection of venoms for immunotherapy.

Methods: 100 patients each with allergic reactions to *Vespula* or honey bee stings and positive i.e. skin tests to the respective venom, were analysed for serum IgE to bee venom, *Vespula* venom and crossreacting carbohydrate determinants (CCDs) by UNICAP (CAP) and ADVIA Centaur (ADVIA). IgE-antibodies to species specific recombinant major allergens (SSMA) Api m1 for bee venom and Ves v5 for *Vespula* venom, were determined by ADVIA. 30 history and skin test negative patients served as controls.

Results: By CAP sensitivity was 1.0 for bee and 0.91 for *Vespula* venom, by ADVIA 0.99 for bee and 0.91 for *Vespula* venom. None of the controls were positive with either test. Double positivity was observed in 59% of allergic patients by CAP, in 32% by ADVIA. sIgE to Api m1 was detected in 97% of bee and 17% of *Vespula* venom allergic patients, sIgE to Ves v5 in 87% of *Vespula* and 17% of bee venom allergic patients. sIgE to CCDs were present in 37% of all allergic patients and in 56% of those with double positivity and were more frequent in bee than in *Vespula* venom allergic patients.

Conclusions: Double positivity of IgE to bee and *Vespula* venom is often caused by crossreactions, especially to CCDs. IgE to both Api m1 and Ves v5 indicates true double sensitization and immunotherapy with both venoms.

U. R. Müller¹, N. Johansen²,
A. B. Petersen², J. Fromberg-
Nielsen², G. Haeberli¹

¹Spital Bern Ziegler, Internal Medicine, Switzerland;
²ALK-Abelló, Horsholm, Denmark

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Prof. Dr. U. R. Müller
Medical Department
Spital Ziegler
Spitalnetz Bern
Morillonstrasse 75-91
CH-3007
Bern
Switzerland

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Hymenoptera venom allergy may cause life-threatening or even fatal systemic allergic reactions (1–3). It is treated successfully by immunotherapy with the responsible Hymenoptera venoms, most often from wasps (*Vespula* spp.) or the honey bee (*Apis mellifera*) (2, 4). Determination of specific serum IgE antibodies to Hymenoptera venoms is a very sensitive diagnostic test for venom allergy (2, 3). However, a large proportion of patients with allergic reactions to honey bee or *Vespula* stings have also specific IgE to the other venom (5–8). Such double positivity (DP) causes significant problems in the selection of venoms for immunotherapy: DP in diagnostic tests may be caused by true double sensitization to both venoms indicating potential systemic allergic reactions to the next sting by either insect species, if not treated by immunotherapy with both venoms. On the other hand, DP can be caused by crossreactions, that occur either on peptide basis, resulting from partial sequence identity of protein allergens in the 2 venoms, e.g. in hyaluronidase (9, 10), or they may be related to so called crossreacting

carbohydrate determinants (CCDs), that are contained in glycoprotein allergens (5–7, 11, 12) of both plant and insect origin. In the case of cross-reactions as a cause for double positivity, the treatment with the venom of the primarily responsible insect alone would be sufficient and more cost efficient. IgE inhibition studies by various methods can help to distinguish double sensitization from cross-reactivity (5–8). They are however often not easy to interpret, rather expensive and not suitable for routine purposes.

In this study, we therefore determined specific serum IgE antibodies to both bee venom (BV) and *Vespula* venom (VV) by two different methods, UNICAP and ADVIA Centaur, in 200 patients with a history of either BV or VV allergy and positive skin tests to the respective venom and analyzed the data for DP. We also looked for sIgE to recombinant species-specific nonglycosylated major allergens (SSMA) from both honey bee and *Vespula vulgaris* to distinguish true double sensitization from cross-reactivity.

Patients and Methods

Patients and controls

One hundred patients with a convincing history of a systemic allergic reaction to a honey bee sting within the last year and positive i.c. skin tests with bee venom and 100 patients with a convincing history of a systemic allergic reaction to a *Vespula* sting within the last year and positive i.c. skin tests to the respective insect were carefully selected from more than 600 Hymenoptera sting allergic individuals referred to our clinic in the years 2000–2002. Patients with documented allergic reactions to stings by both insects were excluded.

Thirty history and skin test negative individuals served as controls.

Skin tests

In all patients and controls, skin sensitivity to both venoms was determined by intradermal skin test end-point titration as described previously (13) with serial tenfold dilutions from 10^{-8} to 10^{-3} g/l. The endpoint concentration (STEP) was defined as the lowest concentration resulting in a wheal of >5 mm in diameter with erythema. A wheal reaction of >5 mm in diameter with surrounding erythema at a venom concentration $\leq 10^{-3}$ g/l was considered as positive. Venoms for skin testing were obtained from ALK-Abello, Horsholm, Denmark.

Specific serum IgE antibodies (sIgE)

In both patients and controls, sIgE to bee venom (BV) and *Vespula* venom (VV) were determined by UNICAP (CAP) (Phadia, Uppsala, Sweden) and ADVIA Centaur (ADVIA) (14) (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). sIgE to CCDs were estimated to MUXF3 from bromelin (Ro214) by CAP and to whole bromelin by ADVIA. Moreover, sIgE were determined to the species-specific recombinant major allergens (SSMA) Api m1 for BV and Ves v5 for VV, by ADVIA. A value of ≥ 0.35 kU/l was considered as positive. Recombinant Api m1 produced in *Escherichia coli* was kindly made available by Prof. Reto Crameri and Kurt Blaser, SIAF Davos, Switzerland (15, 16) and recombinant Ves v5 by ALK-Abello, Horsholm, Denmark (17).

Statistical analysis

Correlation of tests performed with CAP and ADVIA with the same allergens, and between whole BV and Api m1 sIgE, and whole VV and Ves v5 sIgE, were calculated by Spearman's rank correlation test. Frequencies of DP obtained with the two tests, for whole venoms and SSMA, for all patients and BV and VV allergic patients separately, were analyzed by χ^2 -test. Presence of sIgE to Ro214 (CAP) in all patients and BV and VV allergic patients separately, with regard to DP or single positivity, were analyzed by χ^2 -test.

The study protocol was accepted by the ethical committee of the canton of Bern (KEK), Switzerland.

Results

Clinical data on venom allergic patients

All patients had a well-documented history of systemic allergic reactions to either honey bee or *Vespula* stings and positive skin tests to the respective venom. Table 1

Table 1. Degree of severity [2] and diagnostic tests in 100 bee venom and 100 *Vespula* venom allergic patients

Parameter	100 bee venom allergic patients	100 <i>Vespula</i> venom allergic patients
Degree of severity		
Grade I	14	5
Grade II	16	20
Grade III	45	36
Grade IV	25	39
BV i.c. skin test EPC (g/l)		
$> 10^{-3}$	0	53
10^{-3}	1	21
10^{-4}	22	23
$< 10^{-4}$	77	3
VV i.c. skin test EPC (g/l)		
$> 10^{-3}$	45	0
10^{-3}	16	2
10^{-4}	35	59
$< 10^{-4}$	4	39
BV sIgE CAP class (kU/l)		
0 (< 0.35)	0	39
1 (0.35–0.69)	1	12
2 (0.7–3.4)	11	37
3 (3.5–17.4)	29	13
≥ 4 (≥ 17.5)	59	0
VV sIgE CAP class (kU/l)		
0 (< 0.35)	43	9
1 (0.35–0.69)	16	4
2 (0.7–3.4)	30	38
3 (3.5–17.4)	8	40
≥ 4 (≥ 17.5)	3	9

EPC, endpoint concentration.

shows the degree of severity of the systemic allergic reaction according to H.L. Mueller modified by Müller (2) and the results of i.c. skin tests and venom specific IgE antibodies with the two venoms in both BV and VV allergic patients. While the severity of systemic reactions is comparable in the two groups, both skin sensitivity and sIgE to the responsible venom are higher in BV than in VV allergic patients, as previously reported (2). Both skin sensitivity and sIgE to the two venoms were much higher in patients with reactions to the venom of the responsible insect indicated by history than to the other species.

Specific IgE to venoms and CCDs estimated by UNICAP and ADVIA Centaur

Figure 1A and B show sIgE values for both BV and VV obtained with the two methods in the 200 Hymenoptera venom allergic patients. With CAP, 100% of BV and 91% of VV allergic patients and with ADVIA, 99% of BV and 91% of VV allergic patients were positive. The correlation between the two methods was highly significant for both venoms, but somewhat closer for VV allergic patients. On the other hand, none of the history and skin test negative controls had positive sIgE tests with either method or venom (Table 2). Sensitivity and specificity of both tests are thus high and comparable.

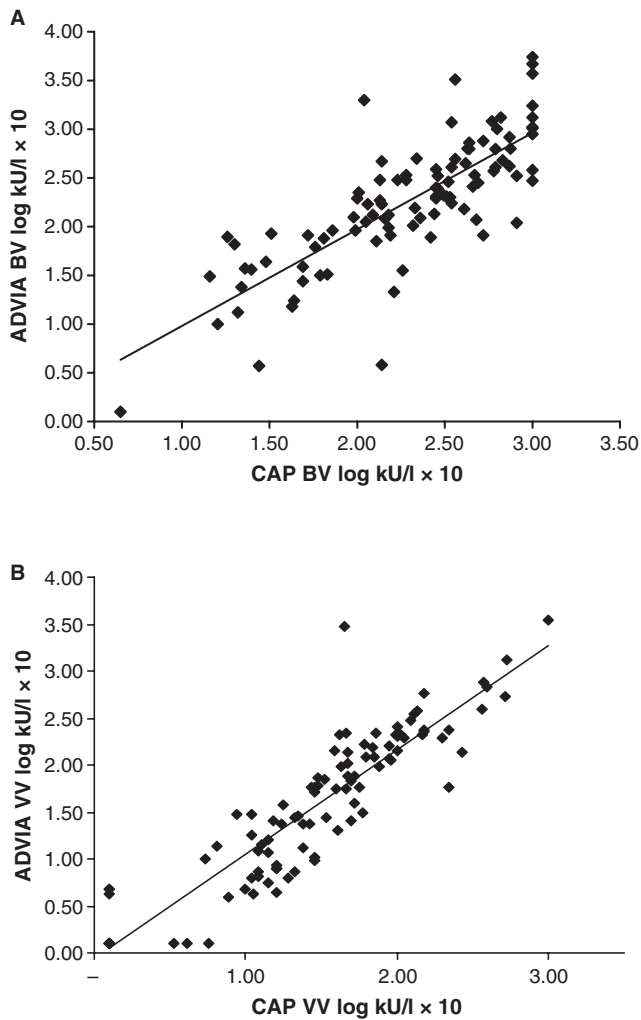


Figure 1. Correlations of serum IgE (sIgE) to bee venom and *Vespula* venom estimated by CAP and ADVIA Centaur (A) sIgE to bee venom (BV): Spearman's rank $r = 0.799$; $P < 0.0001$ (B) sIgE to *Vespula* venom (VV): Spearman's rank $r = 0.913$; $P < 0.0001$.

Table 2. sIgE to BV, VV, Api m1, Ves v5 and CCDs as estimated by UNICAP and ADVIA Centaur

Specific IgE ≥ 0.35 kU/l to	In 100 bee venom allergic patients	In 100 VV allergic patients	In 30 test + history negative controls
BV CAP	100	57	0
ADVIA	99	20	0
VV CAP	61	91	0
ADVIA	44	91	0
Ro214 CAP	50/96 (52)	13/99 (13)	0
Bromelin ADVIA	29/99 (29)	12/99 (12)	6 (20)
Api m1 ADVIA	96/99	17	0
Ves v5 ADVIA	17/99	87	0

Percentage values are given in parenthesis.

sIgE to CCDs as estimated by CAP (Ro214) and ADVIA (bromelin) are also shown in Table 2. With both methods, sIgE to CCDs are found significantly

more often in BV than in VV allergic individuals (CAP $\chi^2 = 34.49$; $P < 0.0001$; ADVIA $\chi^2 = 9.09$; $P < 0.01$). CAP Ro214 (the CCD epitope from bromelin) detects CCD sIgE definitely more often than ADVIA bromelin extract in venom allergic patients, while in non allergic controls CCD sIgE were only detected by ADVIA.

Specific IgE to Api m1 and Ves v5

Tested by ADVIA, sIgE to Api m1 were detected in 96/99 (97%) of BV allergic patients with sIgE to whole BV, sIgE to Ves v5 in 87/91 (96%) of VV allergic patients with sIgE to whole VV, but in none of the 30 controls (Table 2). The correlation between sIgE to whole venom and corresponding SSMA is shown in Fig. 2A and B. It

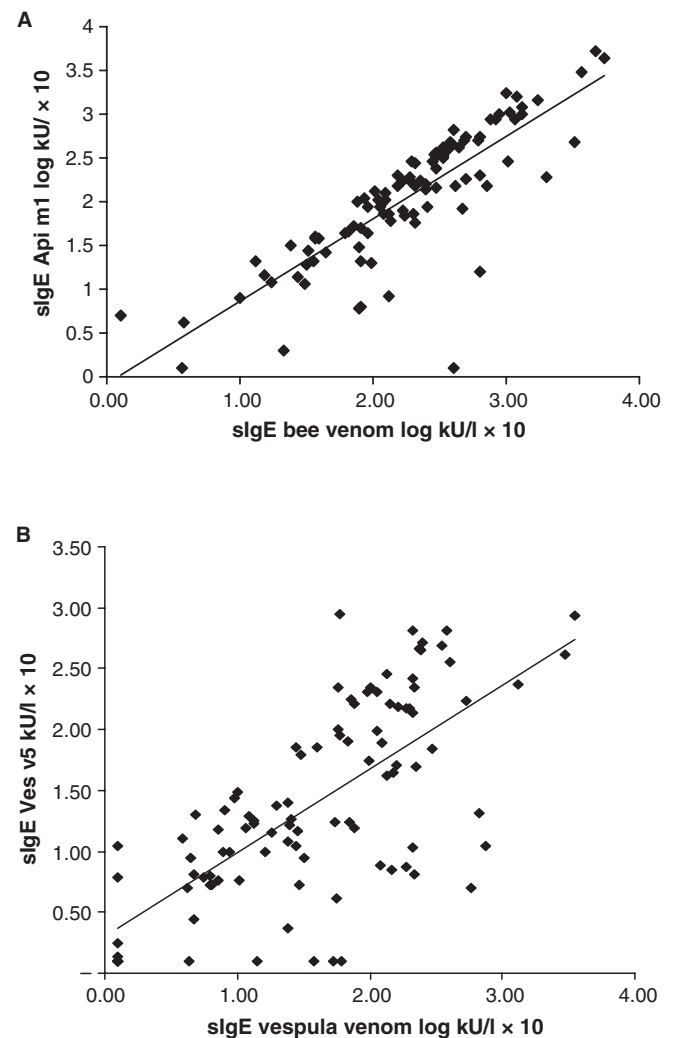


Figure 2. Correlations of serum IgE to whole venoms and species-specific allergens of honey bee and *Vespula* by ADVIA Centaur (A) between sIgE to bee venom and to Api m1: Spearman's rank $r = 0.853$; $P < 0.0001$ (B) between sIgE to whole *Vespula* venom and Ves v5: Spearman's rank $r = 0.675$; $P < 0.0001$.

Table 3. Double positivity for sIgE to BV and VV in 200 venom allergic patients

Double positivity with	In 100 pts allergic to BV	In 100 pts allergic to VV	Double pos total
ST EPC			
≤ 10 ⁻³ g/l	55	47	102 (51)
≤ 10 ⁻⁴ g/l	38	24	62 (31)
CAP			
≥ 0.35 kU/l	61	57	118 (59)
≥ 0.7 kU/l	51	42	93 (46)
DP only in CAP	19	36	55
ADVIA			
≥ 0.35 kU/l	43	20	63 (32)
≥ 0.7 kU/l	32	10	42 (21)
DP only in ADVIA	2	0	2
SSMA ADVIA ≥ 0.35 kU/l	17	17	34 (17)

Percentage values are given in parenthesis.

was significant for both BV and VV allergic patients, but somewhat closer for BV and Api m1 than for VV and Ves v5. sIgE to Api m1 were positive in one BV allergic patient and one VV allergic patient with no sIgE to whole BV. Ves v5 sIgE were detected in 2 VV allergic patients with no sIgE to whole VV.

Double positivity to BV and VV

Double positivity to BV and VV was observed with both methods as shown in Table 3 and was significantly more frequent with CAP than with ADVIA ($\chi^2 = 30.53$; $P < 0.0001$) for all 200 patients and also for patients allergic to BV ($\chi^2 = 6.49$; $P < 0.025$) and patients allergic to VV ($\chi^2 = 50.6$; $P < 0.0001$) separately. In 61 patients, DP was observed with both methods, in 55 only with CAP and in two only with ADVIA. In 34 of the 118 (29%) patients double positive with CAP and in 34 of the 63 (54%) double positive with ADVIA for whole venoms, DP was also found for both SSMA ($\chi^2 = 9.65$; $P < 0.01$). DP was more prevalent in BV than in VV allergic patients with both tests. However, the difference reached significance only in ADVIA ($\chi^2 = 13.625$; $P < 0.001$). sIgE to Ro214 were significantly more frequent in all patients with DP in CAP and in ADVIA, and also for BV and VV allergic patients separately in CAP, but not for BV allergic patients in ADVIA (Table 4).

Table 4. Estimation of CCDs by CAP Ro214 (BV allergic $n = 96$; VV allergic $n = 99$): comparison of prevalence of sIgE to CCDs in double-positive and single-positive patients

Test	Allergic to	Double positivity		Single positivity		Statistics	
		Ro214+	Ro214 -	Ro214+	Ro214 -	χ^2	P
CAP	Honey bee	39	20	11	26	12.05	<0.001
	Vespula	13	42	0	44	11.97	<0.001
	All patients	52	62	11	70	22.22	<0.0001
ADVIA	Honey bee	27	16	23	30	3.58	ns
	Vespula	7	12	6	74	11.59	<0.001
	All patients	34	28	29	104	21.1	<0.0001

In all history and skin test positive BV allergic patients, sIgE to BV was higher than to VV by CAP, while 1/100 BV allergic patient had slightly higher sIgE to VV than to BV (2.01 vs 1.52 kU/l) in ADVIA. Of all VV allergic patients by history and skin test, 6/100 had higher sIgE to BV by CAP but none by ADVIA. The BV allergic patient with slightly higher sIgE to VV by ADVIA and two of the six VV allergic patients with higher sIgE to BV by CAP were positive with both Api m1 and Ves v5 indicating true double sensitization.

Discussion

In patients with systemic allergic reactions to Hymenoptera stings, frequent double positivity to honey bee and *Vespula* venom was first described shortly after the introduction of Phadebas RAST (5) and confirmed in several publications since (6–8, 18–21). A prevalence of 20–50% of DP BV/VV was recorded and a significant increase in DP was observed after the introduction of CAP (22). A similar increase has been reported for food IgE testing in patients with pollen allergy after CAP introduction (23). DP may be caused by true double sensitization indicating immunotherapy with both venoms or by cross-reactions between the two venoms indicating treatment with only the primarily sensitizing venom. Cross-reactions can be caused by partial sequence homology between peptide allergens in both venoms, which according to available data is limited to hyaluronidase (9). Other major venom allergens like phospholipase A₂ from BV or antigen 5 and phospholipase A₁ from *Vespula* venom are structurally not related (10). In spite of the careful selection of BV and VV allergic patients in this study, we found a high percentage of DP in skin sensitivity and especially in venom sIgE as determined by CAP. Even the exclusion of patients with allergic reactions to both insects, however, does not completely exclude true double sensitization as most adults in Switzerland have been stung by both insects and thus could be sensitized to both, even when they did not react to the last sting. Moreover, the reliable identification of all stinging insects from history is not possible in all patients with allergic sting reactions.

Cross-reacting carbohydrate determinants (CCDs), which are frequently present in allergens of both insects and plants, but not of mammals, are contained in several allergens of the two Hymenoptera venoms and have been shown to be responsible for part of the DP (6–8, 11, 12, 21). The relevant carbohydrate epitope was identified as an α -1-3 fucosylated *N*-glycan (12). Definite *in vitro* cross-reactions caused by these CCDs were described between insect allergens and pollen of grass, trees, rape and weeds as well as natural rubber latex, bromelain from pine apple, tomatoes and horse raddish peroxidase (6, 7, 22, 24). Most authors think that CCDs are clinically of little relevance (6, 11, 22). This view is supported by the

observation that in venom allergic patients with sIgE to pollen, but no symptoms of hay fever, there is no increase of sIgE to pollen and CCDs during the pollen season whereas after a sting, sIgE to CCDs and the venom, but not to pollen, increase strongly (22). On the other hand, in one study (7), more than 70% inhibition of sIgE binding to both venoms by horse raddish peroxidase was observed in two patients with a history of severe Hymenoptera sting reactions. Yet we know that the severity of an allergic sting reaction is not related to the serum level of venom sIgE (2, 4) and thus an inhibition of more than 70% by CCDs may not exclude a relevant sensitization to a venom peptide allergen. In another study on tomato allergy, basophil histamine release *in vitro* could be observed after addition of not only tomato allergen but also of isolated CCDs (24). The high number of DP to BV and VV also in skin tests in this study (Table 1 and 3) points to some biologic activity of the CCDs too. However, we know that skin tests to venoms remain positive for years during venom immunotherapy, when patients tolerate stings without any problems (2, 3). A number of different causes for clinically irrelevant sIgE results have been suggested, like levels of total IgE or specific IgG in relation to specific IgE (26). One study indicated that IgE affinity has an influence on basophil activation and therefore likely also on histamine release: Low affinity sIgE had a 1000-fold lower basophil activation capacity than high affinity sIgE (27, 28).

The significantly higher DP rate in BV than in VV allergic patients found in our study (Table 3) may be explained by the fact that all major BV allergens, notably phospholipase A₂, hyaluronidase, acid phosphatase and protease are glycosylated, while the two most relevant VV allergens, Ves v 5 and phospholipase A₁, are not. Hyaluronidase from VV is strongly glycosylated, but is probably only a minor allergen (6). This hypothesis is confirmed by our finding of a much higher rate of sIgE to Ro214 in BV than in VV allergic patients, an observation which, together with the higher DP rate in BV allergic patients, also supports the concept that many cases of DP are caused by CCDs. The higher DP rate in CAP in our study than so far reported (6–8, 20–22) could be because of the fact that BV allergy is more frequent in Switzerland than VV allergy and also that all patients' sera were obtained within a year, most of them within 4 months after the last sting reaction. In a recent publication (22), it was shown that after a sting reaction, sIgE to CCDs increase as sIgE to venom peptides, but decrease more rapidly afterwards.

With regard to the choice of venoms for immunotherapy, DP was so far analyzed by RAST-inhibition tests with both venoms and with CCDs, more recently by immunoblot inhibition with venoms and proteins rich in CCDs (6–8), such as oil seed rape or bromelain. Such inhibition tests are expensive. Moreover, they are sometimes difficult to interpret, especially at sIgE levels lower than class three for both venoms and finally we cannot be

sure that an inhibition of 70–80% excludes sensitization to a peptide epitope completely.

In this study, we estimated sIgE to BV and VV with a new method, ADVIA Centaur (14) in comparison with the well established UNICAP. The difference between the two tests is the following: In CAP, the allergen is bound to the immunosorbent, then incubated in the patient serum whereby all venom-specific antibodies, IgE but also antibodies of other immunoglobulin classes, are bound and finally a labeled anti-IgE antibody is added for detection of bound sIgE. To minimize competition between IgE and nonIgE specific antibodies, a very high amount of allergen is bound to the immunosorbent. In ADVIA, the anti-IgE is bound to paramagnetic particles, then incubated with the patient serum whereby only IgE is bound and then labeled allergen is added. The advantage of this approach is that 1) there is no interference from nonIgE antibodies and 2) much less allergen is needed and the affinity of the sIgE therefore better regarded; low-affinity cross-reacting sIgE like those to CCDs will thus be less considered. Our results (Tables 3 and 4) together with the comparable sensitivity but significantly lower double positivity rates for both venoms in ADVIA than in CAP and lower prevalence of sIgE to CCDs in BV allergic patients in ADVIA, support this hypothesis (14, 28).

Because of the expenses and the sometimes difficult interpretation of inhibition tests, we chose another approach to distinguish between true double sensitization and cross-reactivity in the presence of DP: We estimated sIgE to recombinant, nonglycosylated, species-specific major allergens (SSMA) Api m1 for BV (15) and Ves v5 for VV (17). In contrast to Ves v5, natural Api m1 is glycosylated, but not so the recombinant preparation used in this study and originating from *E. coli*. It has been shown to have comparable allergenic activity to the natural Api m1 in skin tests and *in vitro* sIgE binding (16, 25). The presence of sIgE to both Ves v5 and Api m1 thus proves true double sensitization and indicates immunotherapy with both venoms. The presence of sIgE to only one of the SSMA on the contrary could allow restriction of immunotherapy to the corresponding whole venom. It may however not completely exclude that an isolated sensitization to another species specific allergen is present. The fact that 97% of BV allergic patients with sIgE to whole BV were also Api m1 positive and 96% of those with VV allergy and sIgE to whole VV also to Ves v5, indicates that this risk is minor. True double sensitization was indicated by the presence of sIgE to both SSMA of BV and VV in 34 of 63 (54%) of the patients with DP to both whole venoms in ADVIA, but only 34 of 118 (29%) of those with DP in CAP. None of the 55 patients with DP only in CAP and of the two with DP only in ADVIA had sIgE to both SSMA (Table 3). The higher DP rate in CAP and the significant difference in concordance between whole venom and the corresponding SSMA in CAP and ADVIA are most likely caused by of the much

greater sensitivity of CAP for low affinity cross-reacting IgE antibodies to CCDs.

We conclude that the majority of DP for BV and VV in Hymenoptera venom allergic patients are caused by cross-reactivity based on sIgE to CCDs. Commercial

availability of tests for sIgE to SSMA of both venoms would allow to reduce the need for expensive inhibition tests and thus also the costs of analysis of double positivity with regard to the choice of venoms for immunotherapy.

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