

Original article

In vitro hymenoptera venom allergy diagnosis: improved by screening for cross-reactive carbohydrate determinants and reciprocal inhibition

Background: Immunoglobulin (Ig) E-double positivity for honeybee (HB) and yellow jacket (YJ) venom causes diagnostic difficulties concerning therapeutical strategies. The aim of this study was to clarify the cause and relation of the cross-reactivity in patients with insect venom allergy.

Methods: For this purpose, 147 patients with suspected stinging insect allergy and CAP-IEIA-double positivity were investigated for specific sIgE to additional cross-reactive carbohydrate determinant (CCD)-containing allergens: timothy grass pollen, rape pollen, natural rubber latex (NRL), bromelain, and horseradish peroxidase (HRP). Sera with sIgE to NRL were further investigated with the commercially available recombinant latex allergens. Reciprocal inhibition assays with both venoms and HRP were performed.

Results: About 36 of 147 (24.5%) patients had sIgE to both venoms only. However, 111 of 147 (75.5%) additionally reacted to CCD-carrying allergens. 89 of 111 CCD-reactive sera had NRL-sIgE. In cases where inhibition experiments were performed, the NRL-sIgE binding was completely abolished in the presence of HRP. Only nine of 61 sera were positive for at least one recombinant latex allergen; all of them were negative in history and NRL-skin prick test. In 43 sera containing sIgE to CCD, HRP inhibition revealed unequivocal results: In 28 of 43 (65%) an HRP-inhibition > 70% of sIgE to one venom occurred, pointing out the relevant venom. In three of 43 sIgE proved to be entirely CCD-specific.

Conclusions: Our data indicate that in cases of IgE positivity to both insect venoms supplementary screening tests with at least one CCD-containing allergen should be performed; HRP being a suitable tool for this test. In addition, subsequent reciprocal inhibition is an essential diagnostic method to specify cross-reacting sIgE results.

**U. Jappe¹, M. Raulf-Heimsoth²,
M. Hoffmann¹, G. Burow³,
C. Hübsch-Müller¹, A. Enk¹**

¹Department of Dermatology and Venereology, University of Heidelberg, Heidelberg; ²Research Institute of Occupational Medicine of the Berufsgenossenschaften (BGFA), Ruhr University Bochum, Bochum; ³Sweden Diagnostics, Medical Research, Freiburg, Germany

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Uta Jappe MD, MSc
Department of Dermatology and Venereology
University of Heidelberg
Vosstrasse 2
69115 Heidelberg
Germany

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Up to 5% of the population in Europe and in North America show systemic reactions to hymenoptera stings, mostly honeybee (HB; *Apis mellifera*) and yellow jacket (YJ; *Vespula germanica* and *Vespula vulgaris*; 1). As many patients fail to identify the stinging insect, skin testing and *in vitro* detection of venom-specific immunoglobulin (Ig) E antibodies are the only tools to detect the culprit insect involved in the allergic reaction and are – in addition to the severity of clinical symptoms – used to select the appropriate venom for immunotherapy (2). The sera of up to 40% of patients with hymenoptera venom allergy show *in vitro* reactivity with both, HB and YJ venom (3–5). This IgE positivity to both hymenoptera venoms has been interpreted as true double sensitization leading to immune therapy against both venoms. However, there are other reasons for IgE-double positivity: (i) true independent sensitization (co-sensitization) to differ-

ent allergens, a very rare phenomenon; (ii) immunochemical cross-reactivity because of sequence homologies between allergens from different sources; (iii) cross-reactive carbohydrate determinants (CCD) may be the major cause of *in vitro*-double positivity to both hymenoptera venoms; and (iv) nonspecific absorption of IgE to the allergosorbent, a phenomenon that is particularly relevant when total serum IgE is extremely elevated. CCD, however, are important antigen targets for specific sIgE-antibody binding providing at least two different IgE-binding sites. They are not only widely distributed in plants (pollen, plant food), but also are present in hymenoptera venoms, as invertebrate glycoproteins, as present in hymenoptera venoms, also bear IgE-binding $\alpha(1,3)$ fucose-containing CCD (6). According to Mari (7), 5% of nonallergic individuals have CCD-sIgE antibodies as well as 10% of nonpollen allergic subjects. The

prevalence of CCD-sIgE has been estimated to be 10–15% in patients with grass pollen allergy (8) and increases up to more than 60% in patients with concomitant sensitization to pollen from trees, grasses and weeds (9). The prevalence in patients with allergy to HB venom is 20% (6). About one in four HB venom and one in 10 YJ venom allergics have been demonstrated to be CCD-sIgE-positive (10). Whether these CCD-sIgE antibodies have clinical relevance still remains controversial (11–13). Ebo et al. (14) demonstrated that sensitization to timothy grass and mugwort pollen was found to elicit false-positive IgE for hymenoptera venoms and vice versa.

The aims of this study were to investigate the prevalence of IgE-double positivity for both hymenoptera venoms and of CCD-sIgE antibodies as a cause for this phenomenon to clarify whether there is cross-reactivity or co-sensitization. In addition, to find a suitable screening allergen to detect CCD-sIgE antibodies, and subsequently to investigate, whether the reciprocal CAP-FEIA inhibition with both venoms and/or a CCD-containing allergen might be valuable tools.

Material and methods

Patients

Hymenoptera venom allergic patients with sIgE antibodies to both, HB and YJ venom ($n = 147/710$), were included in this study and evaluated on the basis of their sex, age, atopic diseases, history for insect stings, the severity of the allergic reaction (Mueller grade), and the culprit insect.

Skin tests

Intradermal skin tests were performed with HB and YJ venom (Reless®, ALK Scherax, Hamburg, Germany) by use of serial 10-fold dilutions with concentrations ranging from 0.00001 to 1.0 µg/ml according to recommended guidelines (15, 16).

In vitro allergy diagnostic: detection of hymenoptera venom-specific IgE antibodies

Allergen-specific sIgE to HB venom (i1, venom from *A. mellifera*) and YJ venom (i3, a mixture of venoms from *V. vulgaris*, *V. germanica* and *V. maculifrons*) and subsequently named allergens were measured with the immunoenzymatic CAP-FEIA system (Sweden Diagnostics, Freiburg, Germany) by using the solid-phase ImmunoCAP® technology, according to the manufacturer's instructions. The measuring range of the CAP-FEIA system is 0.35–100 kU/l. Results > 0.35 kU/l were considered as positive.

Screening for sIgE antibodies to CCD-containing allergens

As there was no single test to detect CCD-sIgE, additional allergens were included which are known to contain glyco-epitopes. Sera with sIgE to both hymenoptera venoms were further investigated for sIgE to CCD-containing allergens [whole extracts of timothy grass pollen, rape pollen, bromelain, horseradish peroxidase (HRP), and natural rubber latex (NRL)].

Rape pollen and bromelain are representative CCD-containing allergens, the latter rarely inducing allergic symptoms (9, 17, 18). Horseradish peroxidase was chosen because it is a 'multivalent' glycoprotein (seven glycan chains in contrast to bromelain, which has only one) and because there is no natural exposure to the human organism (19). Two recombinant allergens of timothy grass pollen, rPhl p 1 and rPhl p 5, without glyco-epitopes, were included to allow the differentiation between a genuine grass pollen sensitization and a CCD-sIgE reaction and served as marker allergens for a genuine sensitization to grass pollen (20).

Reciprocal CAP-FEIA inhibition assay

Inhibition of sIgE antibody measurement with venom preparations as inhibitors was performed as follows. Patient serum (50 µl) and inhibitor (10 µl) were preincubated at 4°C overnight prior to the assay in CAP-FEIA. The venoms were obtained from ALK Scherax [Reless® HB venom (*A. mellifera*); Reless® wasp venom (*Vespula* species; 21)]. In a dose-dependent inhibition experiment, the venom concentration (100 µg/ml) proved to be optimal for the inhibition assay with both venoms, for HRP the optimal concentration was 500 µg/ml. The extent of homologous and heterologous venom inhibition was computed as described elsewhere (22). Inhibition values above 70% were considered as indication for extensive cross-reactivity, whereas values below 25% were considered as no inhibition indicating no cross-reactivity (21).

Screening for the relevance of NRL-sIgE antibodies in IgE-double positive sera

Sera with NRL-sIgE were further investigated with recombinant latex components to determine the individual anti-NRL-sIgE profile. The following recombinant NRL allergens coupled to individual ImmunoCAPs were used: rHev b 1, rHev b 2, rHev b 3, rHev b 5, rHev b 6.01, rHev b 6.02, rHev b 8, rHev b 9, and rHev b 11. All of them were produced in *Escherichia coli* and with the exception of rHev b 5 as fusion proteins with a maltose-binding protein (MBP) as described elsewhere (23, 24). MBP coupled on ImmunoCAPs served as a negative control. A questionnaire for NRL allergy and an additional skin test with an NRL extract (ALK Scherax) were used to evaluate the clinical significance. Healthcare workers ($n = 15$) with confirmed NRL allergy served as controls.

Immunoblot analysis

Binding of IgE specific to HB and YJ venom was detected by Western blot analysis and performed according to the manufacturer's instructions (DPC Biermann, Bad Nauheim, Germany). Criteria ranged from 0 to 3: 0, negative; 1, weak positivity; 2, positivity; and 3, strong positivity.

Statistics

Interrelationships and associations between allergen and immune parameters of quantitative data type were examined by using Pearson correlation coefficients and their 95% confidence intervals (CI). Correlations between skin test and venom inhibition outcomes of qualitative data type, e.g. in cases sIgE-positive to both venoms only and, HRP inhibition in sera additionally sIgE-positive to CCD, were statistically assessed by using the standard chi-square test or in the case of low occupancy numbers by using Fisher's Exact Test. Differences between frequencies of positivity, to both venoms only

and positivity to CCD, were statistically assessed by using the standard chi-square test for contingency tables. Therefore, the Western blot data were classified into four positivity classes ranging from 0 to 3. The degree of association between sIgE concentration and severity of the symptoms after hymenoptera sting, characterized by the Mueller grading system leading to categorical data, was statistically analyzed by using the Kruskal–Wallis test and Spearman rank correlations. Joint influences of HB and YJ venom on Mueller grading were examined by using a multivariate proportional odds model. Statistical analyses were performed with the statistical software package SAS 8.2 version (SAS Institute Inc., Cary, NC, USA) and with the SYSTEM R (version 2.2.1).

Results

Patients

The 147 *in vitro*-double positive (DP) patients (68 female and 79 male patients, aged between 5 and 75 years) could be divided into two groups: *group 1*: patients with sIgE positivity only to HB and YJ venom ($n = 36$); *Group 2*: patients with sIgE positivity to all CCD-containing allergens ($n = 111$). Some of them (29 of 111) were additionally sIgE-positive to the recombinant timothy grass pollen components *Phl p 1* and *Phl p 5*. Most patients (130 of 147) have suffered from systemic reactions to hymenoptera stings. A small subgroup of *in vitro* DP patients ($n = 8/147$; 5.4%) reported clinical reactions to stings of both, HB and YJ, indicating the clinical relevance of their *in vitro* sensitization to both venoms.

Thirty patients ($n = 30/147$; 20.4%) suffered from at least one atopic disease (atopic eczema, allergic rhinoconjunctivitis and allergic asthma). The majority, 97 of 147 (66%) were negative for atopic disease history. Total IgE values in kU/l were < 100 in 54 patients; 100–400 in 67; 400–800 in 12 and 800 to > 1000 in eight patients.

In 20 individuals, retrospective history of atopic diseases was no longer available. Seventy patients identified the stinging insect as wasp, 24 as HB, eight patients had developed allergic symptoms to stings of both hymenoptera, and 44 individuals had not been able to identify the culprit insect.

Skin tests

In 123 patients, skin tests with both hymenoptera venoms could be completed, 84 of 123 were positive for both venoms, 16 of 84 revealed positive reactions to identical concentrations of both venoms (Table 1). There were patients who showed a skin test positivity at the highest venom concentration, who had suffered from anaphylactic shock. The most severe reactions occurred after wasp stings (data not shown).

There was a statistically significant positive correlation between HB-sIgE concentration and the severity of the clinical reaction (Mueller grade) to the insect sting (Kruskal–Wallis test: $P = 0.013$); however, this was not

Table 1. Relation between history (culprit insect) and skin test results ($n = 123$ patients who completed the skin tests)

History (culprit insect)	Skin test		
	YJ venom	HB venom	YB plus HB venom
YJ	49/123 (39.8%)	5/123 (4.1%)	6/123 (4.9%)
HB	2/123 (1.6%)	16/123 (13.0%)	3/123 (2.4%)
YJ and HB	1/123 (0.8%)	2/123 (1.6%)	3/123 (2.4%)
No identification	27/123 (22.0%)	2/123 (1.6%)	7/123 (5.7%)
Total	79/123 (64.2%)	25/123 (20.3%)	19/123 (15.4%)

calculated for YJ-sIgE ($P = 0.24$). A multivariate analysis by using the proportional odds model for the joint correlation of HB- or YJ-sIgE with Mueller grade did not reveal a statistically significant result ($P > 0.05$). Reactions of Mueller grade 3 occurred with all history constellations: the mean of the sIgE concentration was higher for HB than for YJ venom, for the two insect venoms or nonidentified insect venom. For each severity grade there are patients who were not able to identify the culprit insect, which underlines the necessity of specific and sensitive diagnostic allergy tests.

Western blot analysis of *in vitro*-double positive sera

Sera of patients with sIgE only against both hymenoptera venoms showed low sIgE concentrations, which is mostly comparable with the intensity of the immunoblot reaction. Sera of patients with sIgE also to CCD-containing allergens generally induced immunoblot reactions of a stronger intensity indicating that CCD-sIgE reactions are also detected by immunoblot (Table 2). Most HB and YJ allergens used in immunoblot are glycosylated. However, melittin from HB venom and vespigen 5 as well as phospholipase do not contain CCDs (25). In total, 136 of 147 sera were sIgE-positive to nonglycosylated vespigen phospholipase, 126 of 147 to antigen 5, indicating that most of the patients were sensitized to vespigen venom, 13 of 147 were reactive to melittin. The IgE-binding intensity to the different allergens is shown in Table 2: Differences between frequencies of positivity to both venoms only (group A) and positivity to CCD (group B) were statistically assessed by using the standard chi-square test for contingency tables when classifying the Western blot data into criteria of positivity ranging from 0 to 3. The differences between groups A and B were statistically significant for all three glycosylated allergens as well as for YJ phospholipase.

Glycosylated HB allergens are much stronger bound by CCD-sIgE-positive sera, in contrast, the intensity of sIgE binding to melittin was less strong. In those eight patients with clinical reactions to both hymenoptera venoms who were sIgE-positive to both venoms, Western blot was not indicative.

Table 2. sIgE binding to relevant hymenoptera venom allergens detected by Western blot in sera of stinging insect allergic patients IgE-positive to both venoms [group 1: sIgE positivity to both venoms only ($n = 36$); group 2: additional sIgE positivity to CCD ($n = 111$)]

Allergen (kDa)	Intensity 0		Intensity 1		Intensity 2		Intensity 3		Chi-square (P -value)
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	
<i>YJ phospholipase (35)</i>	1 (2.8%)	9 (8.1%)	20 (55.5%)	30 (27.0%)	14 (38.9%)	59 (53.2%)	1 (2.8%)	13 (11.7%)	0.01
<i>YJ – Ag5 (23)</i>	5 (13.9%)	16 (14.4%)	15 (41.7%)	35 (31.5%)	16 (44.4%)	59 (53.2%)	0	1 (0.9%)	0.7
<i>YJ hyaluronidase (43–45)</i>	6 (16.7%)	3 (2.7%)	11 (30.6%)	11 (9.9%)	17 (47.2%)	45 (40.5%)	2 (5.6%)	52 (46.9%)	<0.00001
<i>HB phospholipase A2 (16–20)</i>	8 (22.2%)	3 (2.7%)	14 (38.9%)	10 (9.0%)	14 (38.9%)	67 (60.4%)	0	31 (27.9%)	<0.00001
<i>HB hyaluronidase (44)</i>	6 (16.7%)	5 (4.5%)	17 (47.2%)	9 (8.1%)	13 (36.0%)	72 (64.9%)	0	25 (22.5%)	<0.00001
<i>Melittin (2.8)</i>	34 (94.4%)	101 (91%)	1 (2.8%)	5 (4.5%)	1 (2.8%)	5 (4.5%)	0	0	0.8

Criteria ranged from 0 to 3: 0, negative; 1, weak positivity; 2, positivity; and 3, strong positivity.

Hymenoptera venom allergens values expressed in italics are nonglycosylated allergens.

CCD, cross-reactive carbohydrate determinant.

Prevalence of CCD-sIgE antibodies

The majority of *in vitro* DP sera ($n = 111/147$; 75.5%) reacted specifically to CCD-containing allergens, 89 of 111 (80.2%) to NRL, 103 of 111 (93%) to timothy grass pollen extract, 108 of 111 (97%) to rape pollen extract, and 98 of 111 (89%) to both, bromelain and HRP. The sera with sIgE against HRP and bromelain, however, did not reveal identical results, indicating sIgE binding to different CCD-binding sites. Comparison of the different CCD-containing allergens concerning the concentrations of sIgE binding to them did not demonstrate a discrepancy between bromelain, rape pollen, NRL, and HRP (see Table 3, and Pearson correlation coefficient calculation below). Timothy grass pollen seemed to be of minor importance. However, as shown by parallel investigations with *rPhl p 1* and 5, some individuals may additionally be truly sensitized to timothy grass pollen, whereas sensitization to bromelain and HRP is generally uncommon.

The Pearson correlation coefficient (r) for the comparison between the different CCD-containing allergens included in this study concerning the concentrations of sIgE binding to them revealed for both hymenoptera venoms, $r = 0.22$ (CI: 0.037–0.39); for HB and timothy grass pollen antigen, $r = 0.31$ (CI: 0.13–0.47); for timothy

grass pollen and HRP, $r = 0.59$ (CI: 0.34–0.70); for HB and NRL, $r = 0.43$ (CI: 0.27–0.57; Fig. 1A); and for YJ venom and NRL, $r = 0.41$ (CI: 0.24–0.55). The comparison between the CCD-containing allergens NRL and HRP revealed considerably higher correlations, $r = 0.97$ (CI: 0.95–0.98; Fig. 1B), also between NRL and bromelain, $r = 0.97$ (CI: 0.96–0.98); NRL and rape pollen, $r = 0.92$ (CI: 0.89–0.95); HRP and rape pollen, $r = 0.97$ (CI: 0.96–0.98); and HRP and bromelain, $r = 0.98$ (CI: 0.97–0.99).

Specific IgE antibodies to NRL and recombinant NRL components in *in vitro* DP patients and NRL-allergic controls

Eighty-nine of 111 CCD-reactive sera had sIgE to NRL. In a subgroup of 62 sera sIgE reactivity to nine recombinant NRL allergens was tested: 20 of them were positive to at least one recombinant NRL component but only nine of them showed ‘true’ sIgE reactivity (without reactivity to *rHes b 2* and/or MBP) to *rHes b 8* (three of nine) and very low (<0.4 kU/l) to *rHes b 3* (two of nine), *rHes b 5* (one of nine) and *rHes b 6.01* (two of nine). Twenty-nine of 42 returned the questionnaire for NRL allergy. There was no clinical relevance shown for sIgE to NRL in sera of hymenoptera venom allergic patients.

Table 3. Specific IgE responses to different CCD-containing allergens ($n = 111$)

Allergen	Specific IgE (kU/l)		Number of positive response (≥ 0.35 kU/l)	Median of positive response (≥ 0.35 kU/l)
	Median	Range		
Honeybee	3.06	0.37 to >100	111/111 (100%)	3.06
Yellow jacket	4.95	0.45 to >100	111/111 (100%)	4.95
Timothy pollen	1.83	<0.35 to >100	103/111 (92.8%)	2.59
<i>rPhl p 1</i>	<0.35	<0.35 to 81.7	26/111 (23.4%)	3.91
<i>rPhl p 5</i>	<0.35	<0.35 to >100	24/111 (21.6%)	4.87
Rape pollen	1.54	<0.35 to >100	107/111 (96.4%)	1.59
Bromelain	1.14	<0.35 to >100	97/111 (87.4%)	1.3
NRL	0.88	<0.35 to >100	89/111 (80.2%)	1.13
HRP	1.45	<0.35 to >100	99/111 (89.2%)	1.79

Allergens expressed in italics are nonglycosylated allergens of timothy grass pollen.

CCD, cross-reactive carbohydrate determinant; NRL, natural rubber latex; HRP, horseradish peroxidase; IgE, immunoglobulin E.

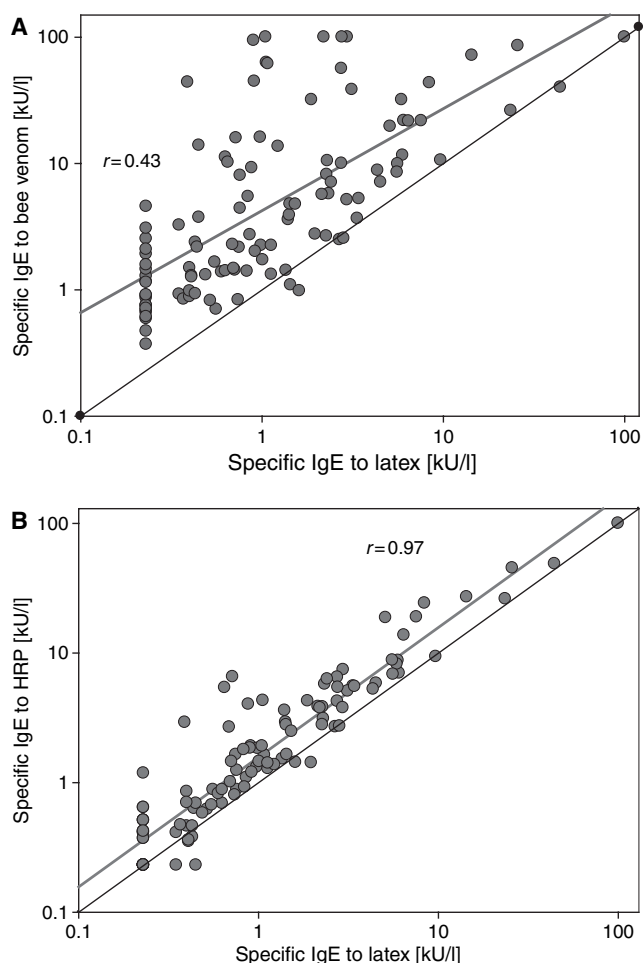


Figure 1. (A) Correlation between the concentrations of natural rubber latex (NRL)-sIgE and honeybee venom sIgE ($n = 108$), the Pearson correlation coefficient being $r = 0.43$ (red line). The bisecting line (black line) indicates that in most of the sera the sIgE concentration to bee venom is higher than the sIgE concentration to NRL. (B) Correlation between the concentrations of NRL-sIgE and the cross-reactive carbohydrate determinant-screening allergen horseradish peroxidase (HRP; $n = 87$), the Pearson correlation coefficient, $r = 0.97$ (green line). The bisecting line (black line) indicates that the sIgE concentrations to HRP are in the majority of the sera slightly higher than to latex.

NRL-sIgE were completely blocked by HRP in the reciprocal inhibition assay, which was in contrast to sera of healthcare workers with confirmed NRL allergy (data not shown). Sera of these 15 healthcare workers did not show any sIgE reactivity to HRP but pronounced sIgE reactivity to rHev b 5 and/or rHev b 6.01 when they were tested against the nine recombinant allergens.

Reciprocal CAP-FEIA inhibition assay

Inhibition of sIgE antibody measurement with venom preparations as well as HRP as inhibitors was performed.

In sera sIgE-positive only to both hymenoptera venoms reciprocal inhibition test with both venoms revealed true double sensitization in 11 of 29 patients.

Reciprocal venom inhibition with CCD-sIgE-containing sera revealed the following results. Whereas, HB venom inhibited YJ venom-sIgE antibodies in 18 of 30 sera with $< 60\%$ blocking, vespid venom partially inhibited HB-sIgE antibodies in 19 of 30 sera but $> 70\%$ in six of 30 sera. Results with only homologous inhibition for both venoms, indicating true double sensitization to HB and YJ venom were not obtained with sera-containing anti-CCD-sIgE. Subsequently, HRP inhibition was performed with CCD-sIgE-containing sera.

Inhibition with HRP in 43 CCD-sIgE-positive sera revealed four groups of inhibition patterns, as illustrated in Fig. 2 are: over 70% HRP inhibition of sIgE to one venom ($n = 28$); 50% HRP inhibition of sIgE to both venoms ($n = 5$); over 70% HRP inhibition of sIgE to both venoms ($n = 3$); and poor HRP inhibition of sIgE to both venoms ($n = 7$). Whereas, 28 of 43 (62.8%) of the sIgE directed against the clinically nonrelevant venom were directed against CCD as shown by 100% inhibition after preincubation with HRP.

Eight patients with confirmed sIgE-double positivity to both hymenoptera venoms had suffered from clinical symptoms after hymenoptera stings from both, HB and YJ, seven of eight had a generalized reaction. Four patients were sIgE-double positive to both hymenoptera venoms only, whereas the other four had sIgE also to CCD-containing allergens. In five of eight, the inhibition assay with both venoms revealed a reciprocal inhibition between 0% and 3%, indicating no cross-reactivity of sIgE between both venoms, and therefore supporting true double sensitization. In two of eight, reciprocal inhibition with both venoms showed cross-reactivity between 5% and 25%, and one serum showed 100% inhibition of YJ-sIgE with HB venom, whereas YJ venom did not inhibit HB-sIgE. Horseradish peroxidase inhibition of sIgE to CCD revealed a 100% HRP inhibition of YJ-sIgE in two of four and of HB-sIgE in one of four, pointing to a possible clinical relevance of CCD-sIgE in these sera. In the fourth serum only minor HRP inhibition was obtained, indicating that CCD-associated cross-reactions did not play a role.

Comparison between intradermal test and HRP inhibition in sera-containing CCD-sIgE antibodies

In 31 of 43 (72.1%) of patients included in reciprocal inhibition, a skin test was performed. In 21 of 31 (68%) the patients reacted to both venoms in the skin test, three of 21 (14.3%) patients reacted to identical venom concentrations. In cases, where sIgE to one venom were blocked by HRP with inhibition values of $> 70\%$ ($n = 28$), suggesting that they were binding only to CCD, 16 of 24 had skin test reactions to both venoms;

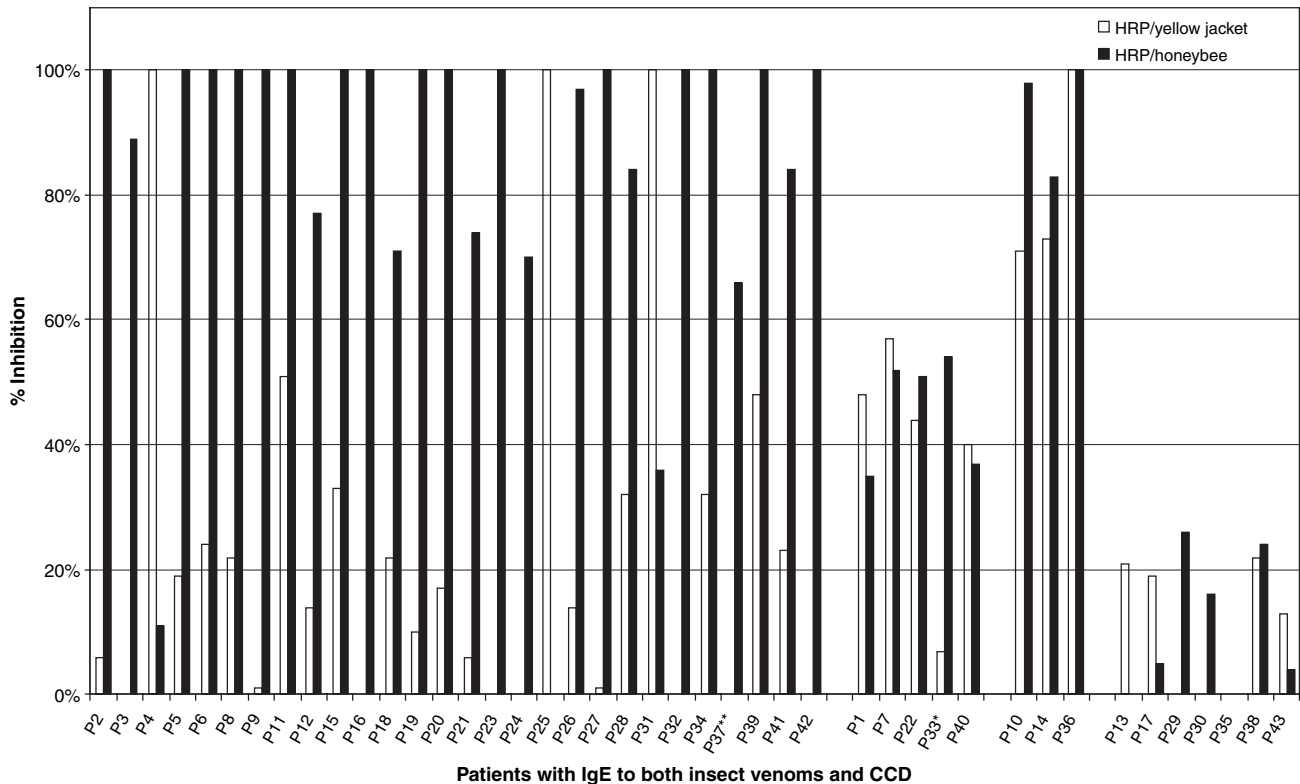


Figure 2. Inhibition with horseradish peroxidase (HRP) in 43 cross-reactive carbohydrate determinant-sIgE-positive sera revealed four groups of inhibition patterns: HRP inhibition >70% of sIgE to one venom ($n = 28$; P2–P42), 50% HRP inhibition of sIgE to both venoms ($n = 5$; P1–P40); complete HRP inhibition of sIgE to both venoms ($n = 3$; P10–P36), and poor HRP inhibition of sIgE to both venoms ($n = 7$; P13–P43). *HRP inhibition of sIgE to one venom 50%, to the other <50%; **HRP inhibition of sIgE to one venom with 66% not exactly >70%, * and **, therefore, not exactly fulfilling the criteria of the concerning group but belonging there rather than into any other of the four groups of inhibition patterns.

however, 22 of 24 pointing to the same venom as the inhibition experiment (Table 4). There is a statistically significant association between the result of the HRP inhibition and the skin test (chi-square test: $P = 0.0045$). This is in particular driven by the 19 patients who show concordantly sensitivity to YJ in both tests.

In addition, also the association between skin test result and reciprocal venom inhibition has been calculated for the group of patients sIgE-positive only to both venoms which was, only just, statistically significant (Fisher's Exact test: $P = 0.048$).

Discussion

In our study, 147 patients with hymenoptera venom allergy were sIgE-positive for both HB and YJ venom, 5.4% reported clinical relevance of their sensitization to both venoms. Egner et al. (3) described no *in vitro* DP patients with clinical relevance, whereas Mueller reported 8.8% (1).

Thirty-six patients were sIgE-positive to both venoms only, and six of 36 (16%) showed independent sensitiza-

tion to both venoms (co-sensitization). This is significantly lower than the percentage rates of 32–67%, reported in the literature: in 50% of the sera a partial cross-reactivity could be shown in addition to sensitization to HB- and YJ-specific epitopes which is higher than previously reported ranges from 14% to 36% (21, 22, 26).

In the present study, 111 of 147 (75.5%) of the included patients showed a strong reactivity with the CCD-containing allergens. Specific IgE to both, HRP and bromelain, were found in 67%, which is in accordance with previous observations (27). The CCD-containing allergens timothy grass pollen, rape pollen, bromelain, and HRP were similarly recognized by sIgE. As the human immune system is not confronted with HRP *in vivo*, a molecule with seven glycan chains, this substance seems to be an applicable screening tool for CCD-sIgE. The detection of CCD-sIgE in patients' sera is highly indicative for *in vitro*-double positivity for hymenoptera venom sIgE as could be shown previously and confirmed by our data (14, 27, 28). The inclusion of a CCD-screening allergen alone in the diagnostic allergy testing; however, is not sufficient to prove the sensitization to one or the other hymenoptera venom, because

Table 4. Comparison of skin test, reciprocal inhibition, and Western blot

Patient†	Skin test results to:		Identification by HRP inhibition*	Accordance: skin test and HRP inhibition	Western blot results			
	YJ (μg/ml)‡	HB (μg/ml)‡			YJ phospholipase	YJ – Ag5	Melittin	Glycosylated venom antigens (YJ and HB hyaluronidase)
3	0.001	0.0001	YJ	No	2	2	0	2
4	0.1	0.0001	HB	Yes	2	1	0	2
5	0.0001	0.1	YJ	Yes	2	2	0	2
8	0.001	1	YJ	Yes	0	2	0	2
9	0.1	1	YJ	Yes	0	0	0	3
11	0.01	0.1	YJ	Yes	2	2	0	3
12	0.001	1	YJ	Yes	2	2	0	3
13	0.0001	0.0001	HB	No	2	1	1	3
15	0.0001	0.01	YJ	Yes	2	2	2	3
17	0.001	0.01	YJ and HB	No	3	1	0	3
18	0.01	1	YJ	Yes	2	2	0	3
20	0.001	0.1	YJ	Yes	2	2	0	3
21	0.001	0.1	YJ	Yes	2	2	0	3
23	0.0001	1	YJ	Yes	2	1	0	2
24	0.0001	0.01	YJ	Yes	2	1	0	2
25	0.01	0.001	HB	Yes	1	0	1	2
26	0.001	0.1	YJ	Yes	2	2	0	3
27	0.001	0.1	YJ	Yes	1	1	0	2
28	0.01	0.01	YJ	No	3	2	0	3
29	1	0.001	YJ and HB	No	2	2	0	3
30	0.0001	0.1	YJ and HB	No	2	2	0	3
31	0.01	0.001	HB	Yes	2	2	0	2
32	0.01	0.1	YJ	Yes	1	2	0	3
33	0.001	0.001	YJ	No	3	2	0	3
34	0.0001	1	YJ	Yes	2	2	0	3
35	0.001	0.0001	YJ and HB	No	2	2	0	2
37	0.0001	1	YJ	Yes	1	0	0	2
39	0.01	0.1	YJ	Yes	3	1	2	3
41	0.001	0.1	YJ	Yes	2	2	0	2
42	0.001	1	YJ	Yes	2	2	0	2
43	0.0001	1	YJ and HB	No	1	1	0	2

YJ, *yellow jacket*; HB, *honeybee*; HRP, horseradish peroxidase.

Western blot results – 0–3 = intensity of immunoblot reaction: 0, negative; 1, weak; 2, moderate; 3, strong.

*Results of complete inhibition of sIgE to one (YJ or HB) or both hymenoptera venoms (YJ and HB).

†Patients as represented in Fig. 2.

‡Venom concentration that induced a positive reaction after intradermal injection.

sensitization to venom-specific proteins is still additionally possible (27, 29) which was also confirmed by our results. As treatment success depends on the choice of the relevant venom, it is important to find the true sensitizer (22). Therefore, additional *in vitro* tests should be performed. The additional reciprocal inhibition with both hymenoptera venoms seems to be a valuable tool (22, 26). However, according to the results of the present study, it is only useful where patients are positive for IgE specific to both venoms only and some rare cases where HRP inhibition with CCD- sIgE-positive sera was not indicative concerning the culprit or nonculprit venom.

Horseradish peroxidase inhibition values above 70% indicating extensive cross-reactivity in sera containing CCD-sIgE mostly were in accordance with skin test results, indicating reliability of the method concerning the detection of the culprit venom. Horseradish peroxidase inhibition of about 50% for both venom sIgEs indicates a

true double sensitization that in 50% is due to both, CCD and protein epitopes, the latter probably being a result of the sequence homology between the hymenoptera venom enzymes, the hyaluronidases and probably further protein epitopes. HB and YJ hyaluronidase, for example, show a sequence homology of between 47% and 57% (30). The hyaluronidases and high-molecular allergens of a molecular weight between 40–100 (HB) and 38–100 kDa (YJ), however, are the most important glycans (29, 31). Phospholipase A2, hyaluronidase, and acid phosphatase are glycosylated (32, 33). However, Western blot analysis revealed that the major CCD-sIgE response is directed against high-molecular glycoproteins (35–95 kDa; 29). Hemmer et al. (29) focused on the unglycosylated venom allergens and found that all wasp allergic patients were positive to antigen 5 in Western blot. This is in contrast to our results: 68 of 126 patients with a positive history for wasp sting were positive for antigen 5-specific IgE. Nearly

a quarter of these patients (37 of 126) had not been able to identify the culprit insect. Not all vespid venom-allergic patients develop sIgE against antigen 5, and only a very small proportion of HB allergic patients have sIgE to HB melittin. As it is still unknown which and how many hymenoptera venom allergens apart from both hymenoptera hyaluronidases and HB phospholipase are glycosylated and whether their immunogenicity is due to peptides or glycans, immunoblot analysis is not helpful to clarify the clinically relevant sensitization.

So far, skin test has been considered as indicative for sensitization, which prompted a comparison between skin test and different *in vitro* tests. In those cases with discrepancy between intradermal test and inhibition this may be because of the fact that the intradermal test is too sensitive to be specific. That severity of reaction and skin test as well as skin test and sIgE detection did not coincide in some individuals is a well-known phenomenon in cases of IgE-mediated hymenoptera venom allergy (34). Skin tests with the venom to which the serum is only reactive because of CCD-sIgE antibodies are according to the literature often negative (29), which is in contrast to our observations. This may be because of the fact that allergens, which are clinically relevant might be different in *in vitro* diagnostic tests and vice versa. Alternatively, the reason for this discrepancy may be found in the way the allergens are presented: either on the solid phase or on mast cells.

Sera with sIgE positivity to CCD-containing NRL were further investigated, including recombinant NRL components and MBP as negative control. Subsequently, the *in vitro* results were evaluated for clinical significance. All *in vitro* results were clinically nonrelevant. IgE responses <0.4 kU/l are borderline and considered to be unspecific. Immunoglobulin E to recombinant NRL components are not protein specific but directed to MBP, the carrier protein, added as control protein in our test system. In addition, from our results it does not seem reasonable to include recombinant NRL components when NRL-sIgE are detected because of CCD binding, the latter being most probably the case when the concentrations of NRL-sIgE and HRP-sIgE as well as sIgE to CCD-containing screening allergens were similar. In cases where inhibition experiments were performed, the NRL-sIgE binding was completely abolished in the presence of HRP, indicating that in hymenoptera venom allergic patients sIgE reactions to NRL mostly because of CCD.

However, there were three sera in which HRP-bound IgE specific to both venoms with inhibition values of 70–100%, suggesting that all venom-specific IgE were reactive only to CCD. Two patients had suffered from a severe clinical reaction after hymenoptera sting indicating that CCD-sIgE may be involved in the development of a manifest allergic reaction. According to several publications, CCD generally are considered to be of no clinical significance, a hypothesis supported by the investigation

of 1000 healthy blood donors who were investigated for sIgE to NRL (35). Until now, no convincing evidence has been reported to support clinical relevance of CCD-sIgE. Patients with antipollen sIgE that exclusively react with CCD are extremely rare. However, a mixed and simultaneous immune response of IgE antibodies in sera of hymenoptera venom allergic patients to glyco-epitopes as well as peptide epitopes in both venoms is still possible, so that even the detection of CCD-sIgE alone does not definitely exclude the simultaneous presence of antipeptide-sIgE (27, 29). So far, CCD-IgE represents one major cause for cross-reactivity and therefore limits the specificity of sIgE tests for insect venoms. However, there are reports suggesting their participation in the induction of clinical symptoms (36, 37 reviewed by Ref. 38) supporting our observation.

In conclusion, our data indicate that in cases of IgE-double positivity to both hymenoptera venoms supplementary CAP-FEIA investigations with at least one CCD-containing allergen should be performed. Horse-radish peroxidase seems to be a valuable screening allergen for the detection of CCD-sIgE and is useful for reciprocal inhibition experiments. Timothy grass pollen and the corresponding recombinant components should be included in cases where patients suffer from additional pollinosis. Whereas reciprocal inhibition with both venoms in sera with sIgE positivity only to both venoms allows the detection of cross-reactivity and true double sensitization, inhibition with HRP is a valuable tool for specifying the venom sensitization in the population with CCD-IgE and should be performed in the first place before including both venoms into the reciprocal inhibition experiment. Only in those cases where sIgE to both venoms as well as to HRP were HRP-inhibited to about 50%, the latter may be valuable. In order to differentiate between true NRL allergy and *in vitro* positivity to CCD, HRP should be added as screening allergen. If recombinant components produced as fusion proteins are used for IgE detection as is the case for most recombinant latex components, MBP should in any case be included in the test as negative control. Since very recently, CCD reagents coupled to an ImmunoCAP matrix, based on the most general CCD glycan structure found in bromelain, MUXF3-type carbohydrate, are commercially available. According to Unger et al., this CCD was even more efficient in inhibitory experiments with sera with confirmed CCD reactivity than the MMXF3-type carbohydrate, a glycan found in HRP (39). MUXF3 CCD ImmunoCAP can be used as CCD-screening tool. As our study demonstrated the value of additional reciprocal inhibition, MUXF3 should also be available as reagent for inhibition procedures. Concerning the question which glycan preparation is the most suitable, more data need to be collected. The fact that HRP contains seven glycan units when compared to bromelain with one glycan unit, favors HRP as screening agent for the detection of sIgE

to different glyco-epitopes, as demonstrated in the present investigation.

The development of recombinant hymenoptera allergens is a possible next step to be taken. From the results of this and other studies it seems to be inevitable to develop and include recombinant allergens in the diagnostic procedures to detect hymenoptera venom allergy. Regarding the fact that a lot of *in vitro* DP patients had an intradermal test positive to both venoms with 19% of them showing reactions to identical titers may indicate that CCDs play a role in skin test reaction as well.

Whether or not CCDs are of clinical relevance still remains to be clarified.

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References

- Mueller U. Insect sting allergy. Stuttgart: Fischer, 1990.
- Ewan PW. Allergy to insect stings: a review. *J R Soc Med* 1985;**78**:234–239.
- Egner W, Ward C, Brown DL, Ewan PW. The frequency and clinical significance of specific IgE to both wasp (*Vespula*) and honeybee (*Apis*) venoms in the same patient. *Clin Exp Allergy* 1998;**28**:26–34.
- Hoffman DR, Miller J, Sutton J. Hymenoptera venom allergy: a geographic study. *Ann Allergy* 1980;**45**:276–279.
- Müller U, Roth A, Yman L, Patrizzi R. Use of RAST technique in wasp sting hypersensitivity. Cross-reactions between various insect antigens are specially considered. *Allergy* 1978;**33**:197–202.
- 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**:259–266.
- Mari A. IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the *in vivo* and *in vitro* reactivity. *Int Arch Allergy Immunol* 2002;**129**:286–295.
- van Ree R, Aalberse RC. Pollen-vegetable food cross-reactivity: serological and clinical relevance of cross-reactive IgE. *J Clin Immunoassay* 1993;**16**:124–130.
- Mari A, Iacovacci P, Afferni C, Barletta B, Tinghino R, Di Felice G et al. Specific IgE to cross-reactive carbohydrate determinants strongly affect the *in vitro* diagnosis of allergic diseases. *J Allergy Clin Immunol* 1999;**103**:1005–1011.
- Kochuyt AM. Insect venom anaphylaxis: from diagnosis to therapy. Thesis. Leuven, Belgium: Medical Faculty, University Leuven, 2000.
- Fotisch K, Altmann F, Haustein D, Vieths S. Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int Arch Allergy Immunol* 1999;**120**:30–42.
- Batanero E, Crespo JF, Monsalve RI, Martin-Esteban M, Villalba M, Rodriguez R. IgE-binding and histamine release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, *Ole e* 1. *J Allergy Clin Immunol* 1999;**103**:147–153.
- Van der Veen MJ, van Ree R, Aalberse RC, Akkerdas J, Koppelman SJ, Jansen HM et al. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J Allergy Clin Immunol* 1997;**100**:327–334.
- Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. *Clin Exp Allergy* 2004;**34**:137–144.
- Nelson HS, Knoetzer J, Bucher B. Effect of distance between sites and region of the body on results of skin prick tests. *J Allergy Clin Immunol* 1996;**97**:596–601.
- Dreborg S. Skin tests used in type I allergy testing (Position paper). *Allergy* 1989;**44**(Suppl. 10):22–33.
- Focke M, Hemmer W, Hayek B, Götz M, Jarisch R. Identification of allergens in oilseed rape (*Brassica napus*) pollen. *Int Arch Allergy Immunol* 1998;**117**:105–112.
- Pike RN, Bagarozzi D, Travis J. Immunological cross-reactivity of the major allergen from perennial ryegrass (*Lolium perenne*), *Lol* p1, and the cysteine proteinase, bromelain. *Int Arch Allergy Immunol* 1997;**112**:412–414.
- Batanero E, Villalba M, Monsalve RI, Rodriguez R. Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J Allergy Clin Immunol* 1996;**97**:1264–1271.
- Valenta R, Vrtala S, Ebner C, Kraft D, Scheiner O. Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. *Int Arch Allergy Immunol* 1992;**97**:287–294.
- Schlenvoigt G, Müller M, Jäger L, Wenz W. *In vitro*-Untersuchungen zum Auftreten von Doppelsensibilisierungen und ihre Charakterisierung bei Insektengiftallergikern. *Allergologie* 1996;**19**:461–464.
- Straumann F, Bucher Ch, Wüthrich B. Double sensitization to honeybee and wasp venom: immunotherapy with one or with both venoms? *Int Arch Allergy Immunol* 2000;**123**:268–274.
- Raulf-Heimsoth M, Rihs HP, Brüning Th. Latex: a new target for standardization. In: Löwer J, Becker WM, Vieths S, editors. Regulatory control and standardization of allergenic extracts, Vol. 94. 10th International Paul Ehrlich Seminar, 2002. Lübeck: Druck- und Verlagshaus Sperlich, Frankfurt am Main, 2003:107–115.
- Raulf-Heimsoth M, Rozynek P, Lundberg M, Cremer R, Brüning T, Rihs HP. Use of recombinant latex allergens. In: Bienenstock JB, Ring J, Togias AGT, editors. Allergy frontiers and futures. Proceeding of the 24th Symposium of the Collegium Internationale Allergologicum, Allergy Clin Immunol Int: J World Allergy Org 2004;**1**:249–251.
- Hemmer W. Cross-reactivity between honey bee and vespidae venoms. A role for hyaluronidases and cross-reactive carbohydrate determinants. *Allergo J* 2005;**14**:553–559.

26. Reisman RE, Wypych JJ, Lazell MI. Further studies in patients with both honeybee- and yellow jacket-venom specific IgE. *Int Arch Allergy Appl Immunol* 1987;**82**:190–194.
27. Kochuyt AM, van Hoeyveld EM, Stevens EAM. Prevalence and clinical relevance of specific IgE to pollen caused by sting induced specific IgE to cross reacting carbohydrate determinants in hymenoptera venoms. *Clin Exp Allergy* 2005;**35**:441–447.
28. Hemmer W, Focke M, Kolarich D, Wilson IBH, Altmann F, Wöhrl S et al. Antibody binding to venom carbohydrates is a frequent cause for double positivity to honeybee and yellow jacket venom in patients with stinging-insect allergy. *J Allergy Clin Immunol* 2001;**108**:1045–1052.
29. Hemmer W, Focke M, Kolarich D, Dalik I, Götz 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**:460–469.
30. King TP, Lu G, Gonzales M, Qian N, Soldatova L. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with hornet and wasp homologs and possible implications for clinical allergy. *J Allergy Clin Immunol* 1996;**98**:588–600.
31. Hoffman DR, Wood CL. Allergens in hymenoptera venom: XI. Isolation of protein allergens from *Vespula maculifrons* (yellow jacket) venom. *J Allergy Clin Immunol* 1984;**74**:93–103.
32. Kubelka V, Altmann F, März L. The asparagines-linked carbohydrate of honeybee venom hyaluronidase. *Glycoconj J* 1995;**12**:77–83.
33. Kubelka V, Altmann F, Staudacher E, Tretter V, März L, Hard K et al. Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A2. *Eur J Biochem* 1993;**213**:1193–1204.
34. Golden DBK, Tracy JM, Freeman TM, Hoffman DR. Insect Committee of AAAAI. Negative venom sting test results in patients with histories of systemic reaction to a sting. *J Allergy Clin Immunol* 2003;**112**:495–498.
35. Ownby DR, Ownby HE, McCullough J, Shafer AW. The prevalence of anti-latex IgE antibodies in 1000 volunteer blood donors. *J Allergy Clin Immunol* 1996;**97**:1188–1192.
36. Foetisch K, Altmann F, Hausteiner D, Vieths S. Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int Arch Allergy Immunol* 1999;**120**:30–42.
37. Foetisch K, Westphal S, Lauer I, Retzek M, Altmann F, Kolarich D et al. Biological activity of IgE specific for cross-reactive carbohydrate determinants. *J Allergy Clin Immunol* 2003;**111**:889–896.
38. Malandain H. IgE-reactive carbohydrate epitopes – classification, cross-reactivity, and clinical impact (2nd part). *Eur Ann Allergy Clin Immunol – Allerg Immunol* 2005;**37**:247–256.
39. Unger E, Holtz A, Brostedt P, Andersson K, Sjölander S, Holquist I et al. Tools for studying cross-reactive carbohydrate determinants. *Allergy Clin Immunol Int: J World Allergy Org* 2005;**17**(Suppl. 1):192.