

ORIGINAL ARTICLE

Cross-reactive carbohydrate determinants strongly affect the results of the basophil activation test in hymenoptera-venom allergy

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Summary

Background In hymenoptera-venom allergy, sera of up to 60% of patients show *in vitro* reactivity to honeybee venom (HBV) and yellow jacket venom (YJV). This phenomenon is mainly caused by specific IgE (sIgE) against cross-reactive carbohydrate determinants (CCD). Whether or not these antibodies can induce clinical symptoms is a longstanding debate.

Objective The aim of this study was to investigate the biological activity of CCD-sIgE and the suitability of the basophil activation test (BAT) in hymenoptera venom-allergic patients having CCD-sIgE.

Methods The biological activity of CCD-sIgE was analysed by application of native and CCD-depleted YJV and HBV in BAT with the blood of 62 hymenoptera venom-allergic patients and 16 non-allergic controls. According to results of intracutaneous skin tests (IC) with YJV and HBV and the existence of CCD-sIgE, patients were classified into six subgroups.

Results In patients with mono-positive IC and CCD-sIgE, and thus double-positive sIgE, BAT with native venoms was also double positive in up to 67% of the patients. In contrast, BAT with CCD-depleted venoms was positive only with the IC-positive venom. However, activation of basophils with the IC-negative venom was significantly lower compared with the IC-positive one. In IC mono-positive patients without CCD-sIgE, BAT was mono-positive with the IC-positive venom in the native and in the CCD-depleted form. CCD-positive patients with double-positive IC were a heterogeneous group, with the majority of CCD-positive patients also being double positive with the native forms of both venoms but mono-positive with the CCD-depleted ones.

Conclusions *In vitro* BAT clearly demonstrates biological activity of CCD-sIgE. However, because most of the patients showed a mono-positive IC and activation of basophils with the IC-negative venom was significantly lower compared with the IC-positive one, the present data suggest that CCD-sIgE is clinically irrelevant in these patients.

Keywords basophil activation test, biological activity, cross-reactive carbohydrate determinants, hymenoptera-venom allergy, venom double positivity

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Introduction

The role of carbohydrate epitopes in the diagnosis and treatment of allergic diseases is still a matter of debate in current allergy research [1, 2]. In a pioneering study, in the early 1980s Aalberse et al. [3] investigated specific IgE antibodies (sIgE) directed against carbohydrates and established the term cross-reactive carbohydrate determinants (CCDs). The main motifs of these carbohydrate epitopes are now known to be *N*-linked glycan chains

with core α 1,3-fucose and β 1,2-xylose residues that form essential parts of two independent epitopes [1]. These carbohydrate structures are widely distributed on glycoproteins from plants and lower animals and occur in natural rubber latex, pollen, vegetables, fruits, hymenoptera venoms, and in some pathogenic worms but not in mammals. According to recent studies, about 15–30% of pollen allergic and 37% of hymenoptera venom-allergic patients display CCD-sIgE [4, 5]. Their ability to elicit allergic symptoms has been investigated in several studies

but is still discussed controversially: while some studies imply that CCD-sIgE is not of clinical significance [4, 6–10], contrasting reports showed that these antibodies have biological activity – providing evidence for possible clinical relevance. Activation of effector cells via CCD-sIgE has been shown, e.g. for carbohydrate epitopes of glycoproteins from celery, tomato, olive tree pollen, cypress pollen, and timothy grass pollen [11–16]. The ongoing debate about the clinical (in)significance of CCD-sIgE was reviewed recently by Altmann [1].

The sera of up to 60% of hymenoptera venom-allergic patients show *in vitro* reactivity with both honeybee venom (HBV) and yellow jacket venom (YJV) [5, 17–20]. The major cause for this phenomenon is supposed to be CCD-sIgE [5, 19]. Minor causes may be real co-sensitization or cross-reactivity due to sequence homology among HBV and YJV proteins, e.g. hyaluronidases [19, 21]. In a recent study by Jappe et al. [19] in 2006, 111 of 147 double-positive patients (75.5%) possessed CCD-sIgE antibodies, whereas only six (4.1%) showed independent sensitization to both venoms.

From the therapeutic point of view, there is a need to discriminate between co-sensitization and cross-reaction, as only patients with co-sensitization need venom immunotherapy (VIT) with both venoms [22]. Because the clinical relevance of CCD-sIgE in hymenoptera-venom allergy is still discussed controversially, the diagnosis and treatment of double-positive patients with CCD-sIgE is also still difficult.

The present study focuses on analysing the biological activity of CCD-sIgE using the basophil activation test (BAT). This test is, due to its high sensitivity and specificity, discussed as an additional diagnostic tool in hymenoptera-venom allergy, especially in patients with a convincing history of a severe allergic reaction but undetectable venom-sIgE and negative skin tests [23–29]. Here, BAT was performed with native, glycosylated vs. CCD-depleted (treated with NaIO₄) YJV and HBV in comparison with skin-test results obtained with both venoms. These findings are expected to provide information on whether CCD-sIgE antibodies might be able to induce allergic reactions. Furthermore, the results obtained will provide insights into whether or not the BAT can be used to identify the appropriate venom(s) for immunotherapy in CCD-positive patients with double-positive sIgE and a mono- or a double-positive skin test.

Methods

Treatment of YJV and HBV with NaIO₄

For the destruction of carbohydrate epitopes, 1 mg of HBV (*Apis mellifera*) or YJV (*Vespula germanica*) (Sigma-Aldrich, Taufkirchen, Germany), dissolved in 0.1 M sodium acetate (Roth, Karlsruhe, Germany), pH 5, was incubated

with 0.01 M NaIO₄ (Merck, Darmstadt, Germany) for 1 or 2 h at room temperature (RT) in the dark. The reaction was stopped by addition of 0.01 M sorbitol (Merck).

SDS-PAGE and Western blot analysis

To verify the destruction of the venoms' carbohydrate epitopes, 10 µg of each venom was subjected to 15% SDS-PAGE [30] and either silver-stained (Silver Stain Plus, Bio-Rad, München, Germany) or transferred onto Immobilon-P Transfer membranes (Millipore, Bedford, MA, USA). The membrane was saturated with 10% bovine serum albumin (BSA) (PAA, Pasching, Austria) in Tris-buffered saline (TBS) with 0.05% Tween20 and 0.2% Triton (T-TBST) overnight at 4 °C, and then washed with T-TBST. Rabbit horseradish peroxidase (HRP)-specific antiserum (Sigma-Aldrich) diluted 1 : 7500 in 5% BSA-T-TBST (incubation for 1 h at RT) was used for the detection of the carbohydrate epitopes of venom glycoproteins. HRP-labelled donkey anti-rabbit IgG (Amersham Life Science, Buckinghamshire, UK) diluted 1 : 10 000 was used as the secondary antibody. Detection was performed using Roti-Lumin (Roth).

Patients

The study protocol was approved by the local ethics committee and all patients enrolled gave their informed consent. The BAT was performed in 62 patients with a clear diagnosis of hymenoptera-venom allergy. Blood samples were taken before VIT. Sixteen healthy, non-allergic subjects with absent sIgE to YJV, HBV, HRP and MUXF (N-glycan chains from bromelain; all measured with UniCAP100, Phadia, Freiburg, Germany), and without a history of hymenoptera stings, served as controls.

Skin tests

Intracutaneous skin tests (IC) with 10-fold serial dilutions of HBV and YJV (ALK SQ, ALK-Scherax, Wedel, Germany), in concentrations ranging from 0.0001 to 1 µg/mL, were performed in all hymenoptera venom-allergic patients but not in control subjects. The IC was considered positive when the weal reaction exceeded a diameter of 5 mm and additional surrounding erythema [31]. Skin-prick tests (SPT) with HRP were performed as described previously [4] using 500 µg/mL of the purified plant glycoprotein (Sigma-Aldrich) in 0.9% NaCl-glycerine (1 : 1), and were considered positive when the weal diameter was ≥ 3 mm. 0.9% NaCl was used as a negative control and histamine (0.1 mg/mL for IC; 10 mg/mL for SPT) as a positive control.

Determination of total and sIgE

Total and sIgE antibodies were measured using Uni-CAP100 (Phadia) according to manufacturer's instructions using Caps with HBV (i1), YJV (i3), HRP (o400), and MUXF from bromelain (o214). sIgE values >0.35 kU/L were considered positive.

BAT

BAT was performed as described earlier with minor modifications using a CD203c-specific antibody (Beckman-Coulter, Krefeld, Germany) [32]. In brief, heparinized whole blood was incubated for 15 min at 37 °C with 10-fold serial dilutions in phosphate-buffered saline (PBS) ($5\text{--}5\times 10^{-4}$ µg/mL) of the native or CCD-depleted hymenoptera venoms. To investigate the ability of other carbohydrate sources to activate basophils, HRP (Sigma-Aldrich) was included in the test panel (also from 5 to 5×10^{-4} µg/mL). To confirm cell responsiveness, 1 µg/mL of a monoclonal anti-IgE antibody (clone BE5; Eurobio-Sciences, Friesoythe, Germany) served as a positive control. After cell stimulation, the reaction was stopped by addition of 20 mM EDTA in PBS and centrifugation at 400 g. Basophils were stained with 10 µL anti-CD203c-PE for 45 min at RT in the dark. Using the whole blood lysing reagent (Beckman-Coulter), erythrocytes were lysed according to the manufacturer's instructions. After washing and resuspension of the cells in PBS with 1% BSA, 60 000 total cells were measured using a FACSCalibur flow cytometer and CellQuestPro software (Becton Dickinson, Heidelberg, Germany).

Because resting as well as activated basophils express the marker CD203c on their cell surface, total basophils could be identified by an increase in fluorescence in the FL-2 channel and were marked (R1). As activated basophils express CD203c to a higher extent compared with resting basophils, they could be identified by a stronger fluorescence increase and were subsequently marked with a second region (R2). CD203c up-regulation in percentage was afterwards determined by displaying total basophils in a histogram gated for R1 (100%) and marking the CD203c^{high} cells with the same settings as R2. The percentage of activated basophils was calculated by subtracting spontaneous CD203c expression (negative control, PBS) from the data obtained with anti-IgE and allergen stimulation; values <0 were set = 0. Stimulation was considered positive when activation of basophils was $>10\%$ after subtraction of the negative control.

In addition to analysis of basophil activation by comparing the resulting single stimulation curves, in skin-test mono-sensitized patients, the areas under the curve (AUC) were also determined and compared. The advantage of the AUC calculation is the reflection of not only the height of the stimulation curve but also the curve progression and

the threshold concentration for the activation of basophils in one value.

AUC calculation and statistical analyses

AUC determinations using the trapezoidal rule as well as all statistical calculations were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

For statistical analyses, the Mann-Whitney test was applied when significance was assessed between CCD-negative and CCD-positive patients; the Wilcoxon's signed-rank test was used when determining the significance between two parameters in one group. Differences were considered as significant at a P -value ≤ 0.05 . All data were expressed as medians (25%, 75% percentiles), unless otherwise stated.

Results

Modification of YJV and HBV

Modification of YJV and HBV was controlled by SDS-PAGE and Western blot using a carbohydrate-specific anti-HRP antiserum. Periodate treatment completely destroyed all YJV and HBV carbohydrate epitopes while the protein backbone remained intact (Figs 1a and b).

Classification and characterization of patients

Based on the skin tests performed with YJV and HBV, patients were classified into three groups (Table 1): one group was mono-sensitized to YJV (positive YJV-IC;

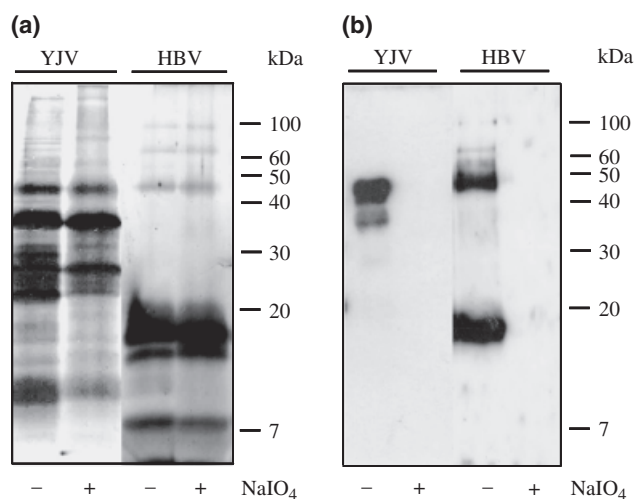


Fig. 1. Effective destruction of carbohydrate epitopes in hymenoptera venoms by periodate treatment. Ten microgram of native yellow jacket venom (YJV), native honeybee venom (HBV), NaIO₄-treated YJV, and NaIO₄-treated HBV were subjected to 15% SDS-PAGE and (a) silver-stained or (b) transferred onto a PVDF membrane and detected using a carbohydrate-specific anti-horseradish peroxidase antiserum.

Table 1. Serological data and skin-test results of the patients investigated

Patient	Sex	Age	Culprit insect	Skin test			Specific IgE (kU/L)				Total IgE	
				YJV*	HBV*	HRP	YJV	HBV	HRP	MUXF		
Positive YJV-IC												
CCD negative												
BW-09	F	47	YJV	1	–	NP	0.70	0.03	0.00	0.00	15.7	
BW-13	M	20	NI	0.1	–	NP	16.40	0.19	0.07	0.05	125	
BW-14	M	43	YJV	0.1	–	NP	31.60	0.07	0.00	0.00	178	
BW-22	M	31	YJV	0.01	–	NP	87.70	0.20	0.01	0.02	396	
BW-59	F	37	YJV	0.01	–	NP	4.05	0.03	0.02	0.00	168	
BW-60	M	42	YJV	0.01	–	NP	59.20	0.17	0.11	0.08	151	
BW-75	F	64	YJV	0.01	–	–	56.90	0.03	0.00	0.01	664	
BW-81	M	66	YJV	0.1	–	–	3.21	0.01	0.00	0.00	72.3	
BW-82	M	28	NI	0.01	–	–	3.42	0.04	0.00	0.01	29.1	
Median							16.40	0.04	0	0.01	151	
CCD positive												
BW-04	M	54	YJV	1	–	NP	7.63	5.81	1.47	2.71	242	
BW-23	M	56	YJV	1	–	NP	4.27	2.93	2.56	2.01	71	
BW-26	F	66	YJV	0.1	–	NP	8.13	8.31	3.96	5.05	98.7	
BW-28	M	25	NI	0.1	–	–	27.60	9.16	1.26	1.12	796	
BW-29	M	51	YJV	0.1	–	NP	14.30	4.13	2.35	1.43	329	
BW-30	F	32	YJV	0.01	–	NP	74.30	4.94	2.98	0.74	302	
BW-32	M	25	YJV	1	–	+	9.92	15.80	9.33	6.70	1000	
BW-39	F	51	NI	0.1	–	+	14.80	3.13	2.28	1.07	68.5	
BW-48	M	51	YJV	0.1	–	NP	23.30	0.59	0.62	0.51	391	
BW-51	M	20	YJV	0.01	–	NP	21.90	1.75	1.43	1.15	324	
BW-54	F	21	NI	0.1	–	NP	12.50	3.39	2.61	2.76	72.1	
BW-66	F	70	YJV	0.1	–	NP	3.53	1.46	0.62	0.43	81.8	
BW-67	M	59	YJV	1	–	NP	2.37	1.31	0.76	0.66	137	
BW-68	M	19	YJV	0.1	–	NP	89.70	3.23	0.58	0.82	663	
BW-71	M	63	YJV	0.01	–	–	65.60	1.78	0.95	0.35	415	
BW-83	F	59	YJV	0.1	–	+	15.90	3.15	1.80	0.87	901	
BW-84	M	18	YJV	1	–	–	22.80	23.00	19.40	17.30	196	
BW-85	M	56	YJV	0.01	–	–	33.10	5.81	3.63	5.67	156	
BW-88	M	26	HO	0.1	–	+	6.87	4.64	4.36	2.08	193	
BW-91	M	63	YJV	0.01	–	+	21.60	10.50	4.16	3.03	355	
Median							15.35	3.76	2.32	1.29	272	
Positive HBV-IC												
CCD negative												
BW-12	F	48	HBV	–	0.01	NP	0.07	22.1	0.18	0.05	63.8	
BW-47	M	34	HBV	–	0.1	NP	0.25	3.93	0.02	0.02	584	
BW-74	M	38	HBV	–	0.1	–	0.05	12.0	0.15	0.15	83.8	
BW-100	M	18	HBV	–	0.1	–	0.02	10.9	0.08	0.05	83.6	
BW-103	M	12	HBV	–	0.01	–	0.08	11.8	0.23	0.07	281	
BW-106	F	64	HBV	–	1	–	0.00	5.14	0.01	0.13	38.8	
Median							0.06	11.35	0.12	0.07	83.7	
CCD positive												
BW-35	F	79	HBV	–	0.1	NP	2.59	71.4	3.9	1.72	143	
BW-42	F	44	HBV	–	0.1	NP	2.18	9.52	3.05	1.4	41.6	
BW-44	F	38	HBV	–	0.01	NP	0.61	20.1	1.16	1.43	88.6	
BW-49	M	58	HBV	–	0.01	NP	2.65	5.70	4.62	3.70	976	
BW-70	M	71	HBV	–	0.001	+	0.45	76.8	1.04	1.21	192	
BW-79	M	47	HBV	–	0.1	–	0.40	58.0	0.86	0.38	123	
BW-93	F	43	HBV	–	0.01	–	1.94	41.9	0.82	0.20	356	
BW-104	M	32	HBV	–	0.01	+	0.30	10.4	1.17	0.83	41.4	
BW-105	F	37	HBV	–	1	–	0.22	9.46	0.65	0.49	32.0	
Median							0.61	20.1	1.16	1.21	123	

Table 1. continued

Patient	Sex	Age	Culprit insect	Skin test			Specific IgE (kU/L)				Total IgE
				YJV*	HBV*	HRP	YJV	HBV	HRP	MUXF	
Double-positive IC											
CCD negative											
BW-25	M	45	HBV	0.1	0.01	NP	0.83	15.7	0.27	0.14	119
BW-45	M	50	NI	1	0.01	NP	2.71	10.0	0.28	0.16	80.9
BW-61	F	59	HBV	0.1	0.1	NP	1.36	4.38	0.02	0.01	65.2
BW-94	F	47	YJV	0.1	0.01	—	5.02	2.13	0.05	0.02	43.3
BW-96	M	19	HBV	1	0.01	—	0.55	13.2	0.04	0.01	68.4
Median							1.36	10.0	0.05	0.02	68.4
CCD positive											
BW-11	F	62	NI	0.1	0.001	NP	0.79	38.8	1.11	0.49	201
BW-33	M	53	YJV	1	1	NP	15.3	3.86	1.00	0.26	131
BW-52	F	73	YJV	0.01	1	NP	> 100	92.0	47.5	53.0	880
BW-62	M	31	NI	0.1	0.1	NP	7.22	4.91	3.04	2.17	182
BW-64	M	43	YJV	0.01	1	NP	7.08	7.28	0.95	1.02	104
BW-65	M	41	YJV	0.1	0.1	NP	2.53	4.65	2.55	4.54	93.5
BW-72	M	40	YJV	0.01	1	+	13.9	1.94	1.52	0.65	148
BW-80	F	61	HBV	1	1	+	2.83	8.48	1.47	1.01	22.9
BW-87	F	65	YJV	1	1	+	8.77	4.83	3.52	3.16	50.6
BW-89	M	41	YJV	0.1	1	+	19.5	17.9	8.47	4.9	424
BW-98	M	28	HBV	0.01	0.01	—	65.6	13.3	0.46	0.71	391
BW-99	F	62	NI	0.01	1	+	8.84	6.86	6.56	4.6	38.3
BW-101	M	42	NI	1	0.001	+	56.8	> 100	79.0	47.8	238
Median							8.84	7.28	2.55	2.17	148

*Venom concentration in µg/mL that induced a positive reaction after intracutaneous injection; concentrations up to 1 µg/mL were applied.

F, female; HBV, honeybee venom; HO, hornet; HRP, horseradish peroxidase; IC, intracutaneous skin test; M, male; MUXF, N-glycan chains from bromelain (provided as Cap o214 by Phadia); NI, not identified; NP, not performed; YJV, yellow jacket venom.

$n = 29$), a second group was mono-sensitized to HBV (positive HBV-IC; $n = 15$), and a third group was sensitized to both YJV and HBV (double positive IC; $n = 18$). According to the results of sIgE measurements obtained with HRP and MUXF (N-glycan chains from bromelain), each of the patient groups was subsequently divided into two subgroups (Table 1): a CCD-negative group with a negative result to both HRP and MUXF (9/29, 6/15, 5/18) and a CCD-positive group with a clear positive result to one or both of the glycoproteins (20/29, 9/15, 13/18). For an overview of the classified subgroups, see also Fig. 6.

All patients with positive YJV-IC possessed YJV-sIgE, and CCD-positive patients in this group additionally displayed HBV-sIgE (median: 3.76 kU/L); thus, they were sIgE-double positive. Most YJV-IC-positive patients reported anaphylaxis due to a yellow jacket sting (23/29), one to a hornet sting, and five had been unable to identify the stinging insect (Table 1).

Vice versa, all patients with positive HBV-IC possessed HBV-sIgE. Similar to the patients with positive YJV-IC, CCD-positive patients in this group were also sIgE-double positive (median YJV-sIgE: 0.61 kU/L). All patients in this group reported anaphylaxis due to honeybee stings.

All patients with a double-positive IC were also double positive with YJV and HBV in sIgE measurements, irrespective of whether they were CCD negative or CCD positive. Eight of these patients reported anaphylaxis due to YJV stings and five due to HBV stings.

BAT in patients with mono-positive IC reveal biological activity of CCD-sIgE

Stimulation of basophils was performed with native YJV (glycosylated), YJV+NaIO₄ (CCD depleted), native HBV (glycosylated), and HBV+NaIO₄ (CCD depleted). All 16 healthy, non-allergic subjects showed no activation of basophils upon stimulation with either native or modified venoms or with HRP, but they were activated by the positive control, i.e. anti-IgE antibody (data not shown).

In Fig. 2, the BAT of one CCD-positive patient with a positive YJV-IC is shown as an example. In this case, there is a clear activation of basophils with native, but not with CCD-depleted HBV, implying that carbohydrate epitopes are responsible for the activation of basophils. This finding is also confirmed by analyses of stimulation curves of CCD-positive patients of both IC mono-positive groups: the CCD-positive patients exemplarily shown in Fig. 3

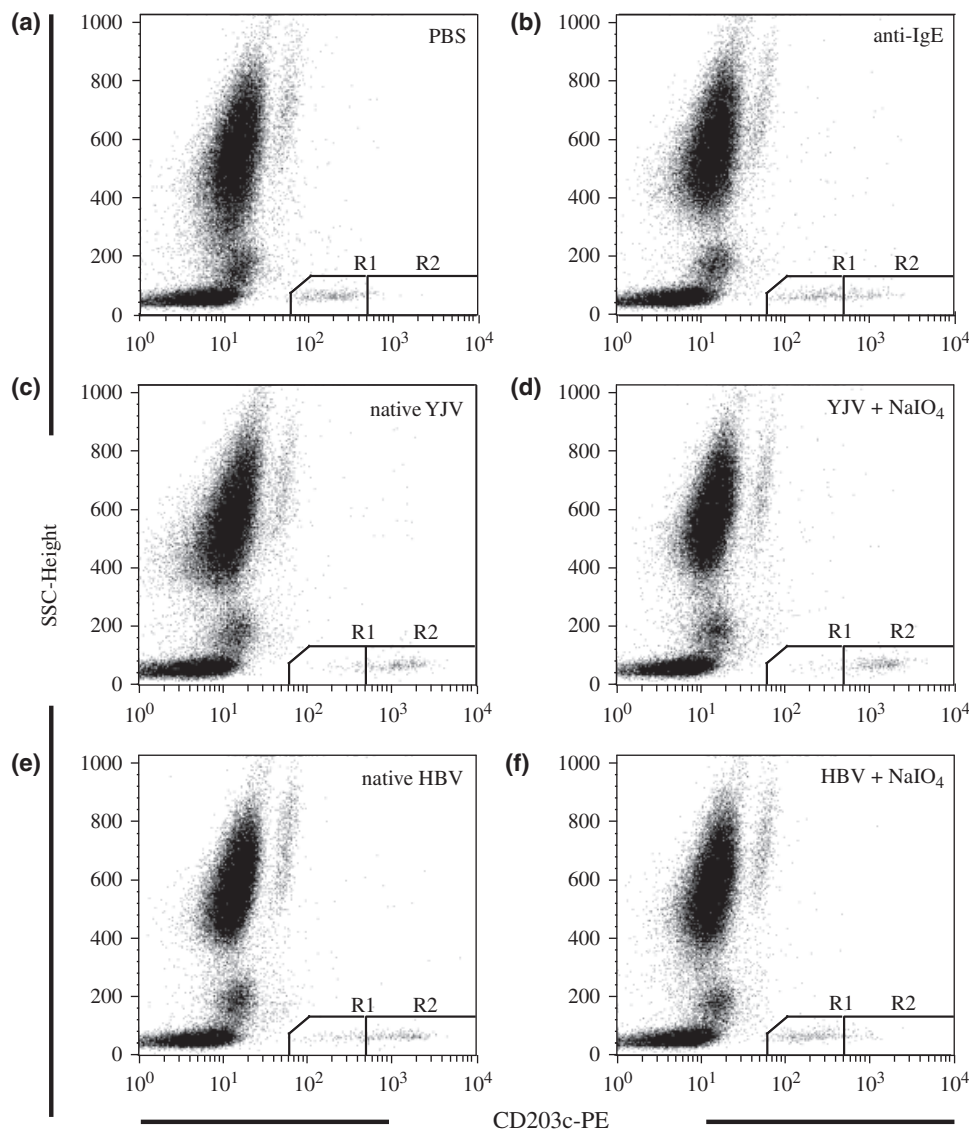


Fig. 2. CD203c expression on basophils up-regulated by allergen or anti-IgE. Data are shown for one cross-reactive carbohydrate determinant-positive patient with a positive yellow jacket venom (YJV) intracutaneous skin test (patient BW-26); the number of activated basophils is presented in parentheses. (a) phosphate-buffered saline (5.4%), (b) anti-IgE antibody (33.8%), 5 μ g/mL of (c) native YJV (85.6%), (d) NaIO₄-treated YJV (88.8%), (e) native honeybee venom (HBV) (69.7%), or (f) NaIO₄-treated HBV (14.2%).

(Fig. 3b: BW-26, positive YJV-IC; Fig. 3d: BW-35, positive HBV-IC) clearly revealed basophil activation with the native, glycosylated venom that was negative in the skin test (BW-26: HBV; BW-35: YJV), but no activation with the corresponding CCD-depleted venom. Calculated AUC values also reflect this fact: the AUC values for the native and CCD-depleted HBV of BW-26 are 59.65 and 10.63, and for native and CCD-depleted YJV of BW-35, they are 43.19 and 9.79. In contrast, basophil activation was similar between the native and CCD-depleted form of the venom that induced the positive skin reaction (Fig. 3b: YJV, Fig. 3d: HBV). In patients without CCD-sIgE, only the IC-positive venom was also positive in the BAT, irrespective of whether it was glycosylated or not (Fig. 3a: BW-14,

AUC for native and CCD-depleted YJV: 166.54 and 172.83, and Fig. 3c: BW-103, AUC for native and CCD-depleted HBV: 86.03 and 80.18).

In both skin-test mono-positive patient groups, the BAT results of all CCD-negative and CCD-positive patients were also investigated by comparing the calculated AUC values for each allergen (Fig. 4). Similar to the results shown exemplarily in Fig. 3, CCD-negative and CCD-positive patients with positive YJV-IC showed a strong, although not equally strong, reaction to both native and CCD-depleted YJV (Fig. 4a). In contrast, native HBV activated basophils from CCD-positive patients, but not basophils from CCD-negative patients, although activation with native HBV was significantly lower compared

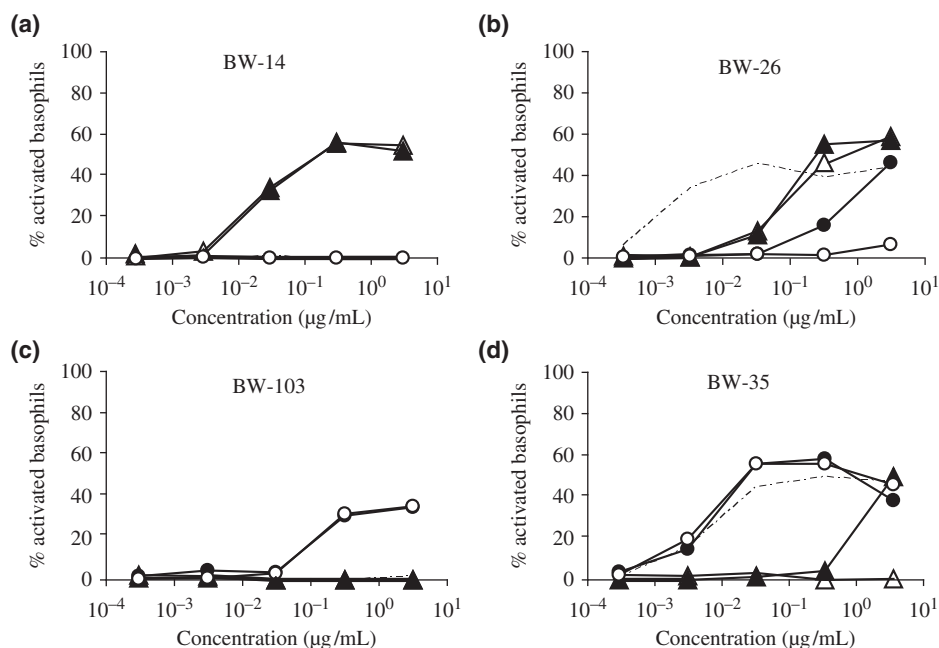


Fig. 3. Basophil activation in representative patients with a mono-positive intracutaneous skin test. (a) Cross-reactive carbohydrate determinant (CCD)-negative patient with a positive yellow jacket venom (YJV) intracutaneous skin test (IC). Area under the curve (AUC) value for native YJV: 166.54, NaIO₄-treated YJV: 172.83, native honeybee venom (HBV): 0.48, NaIO₄-treated HBV: 0.94, and horseradish peroxidase (HRP): 1.26. (b) CCD-positive patient with positive YJV-IC. AUC value for native YJV: 136.27, NaIO₄-treated YJV: 128.56, native HBV: 59.65, NaIO₄-treated HBV: 10.63, and HRP: 205.73. (c) CCD-negative patient with positive HBV-IC. AUC value for native YJV: 2.28, NaIO₄-treated YJV: 4.27, native HBV: 86.03, NaIO₄-treated HBV: 80.18, and HRP: 5.15. (d) CCD-positive patient with positive HBV-IC. AUC value for native YJV: 43.19, NaIO₄-treated YJV: 9.79, native HBV: 210.91, NaIO₄-treated HBV: 218.12, and HRP: 189.85. ▲, native YJV; △, NaIO₄-treated YJV; ●, native HBV; ○, NaIO₄-treated HBV; –, HRP.

with native YJV ($P < 0.001$; Fig. 4a). CCD-depleted HBV barely activated basophils from either group of patients. The differences in basophil activation with native and CCD-depleted HBV in the CCD-positive group were highly significant ($P < 0.001$; Fig. 4a). These data clearly demonstrate that carbohydrate epitopes are able to mediate basophil activation in hymenoptera-venom allergy, indicating that the CCDs are the reason for the BAT double positivity.

In patients with positive HBV-IC (Fig. 4b), the same results were observed, only vice versa: here, basophils of CCD-negative and CCD-positive patients showed a strong reaction upon stimulation with both native and CCD-depleted HBV. CCD-positive patients additionally revealed an activation after stimulation with native but not with CCD-depleted YJV ($P < 0.05$), while basophils of CCD-negative patients were not activated with either form of YJV. The differences between CCD-negative and CCD-positive patients in basophil activation after stimulation with native YJV were statistically significant ($P < 0.05$). Comparison of activation obtained with native HBV and native YJV in CCD-positive patients revealed significantly higher activation with native HBV ($P < 0.01$; Fig. 4b).

The activation of basophils achieved with HRP in CCD-negative patients of both skin-test mono-sensitized groups was marginal, whereas basophils in the CCD-positive groups were strongly activated. The differences

observed in both groups with mono-positive IC are highly significant ($P < 0.001$, Fig. 4a; $P < 0.01$, Fig. 4b).

A more detailed picture emerges when the thresholds for activation with the different allergens in the BAT are compared between CCD-negative and CCD-positive patients of both skin-test mono-sensitized groups (Fig. 5). For most patients, the threshold for activation with the insect venom that was also positive in the skin test was in the range of 0.05–0.5 $\mu\text{g/mL}$ (native YJV, Fig. 5a; native HBV, Fig. 5b). When stimulating basophils with the other native venom, none of the CCD-negative patients with positive YJV-IC (native HBV, Fig. 5a) and only one of the patients with positive HBV-IC (BW-12; native YJV, Fig. 5b) was positive. In contrast, more than half of the CCD-positive patients (50% with positive YJV-IC; 67% with positive HBV-IC) showed activation, although at 10–100-fold higher thresholds; typically in the range of 0.5–5 $\mu\text{g/mL}$ for patients with positive YJV-IC (native HBV, Fig. 5a) and at 5 $\mu\text{g/mL}$ for patients with positive HBV-IC (native YJV, Fig. 5b). When stimulated with HRP, all CCD-negative patients except for one (BW-12, Fig. 5b) showed no response (Figs 5a and b). In CCD-positive patients, the activation threshold for HRP ranged from 5×10^{-4} to 5 $\mu\text{g/mL}$, with a maximum value at 0.05 $\mu\text{g/mL}$. However, 10–20% of the CCD-positive patients showed no response to HRP (HRP, Figs 5a and b). An overview of the IC mono-positive subgroups identified by BAT analysis is shown in Fig. 6.

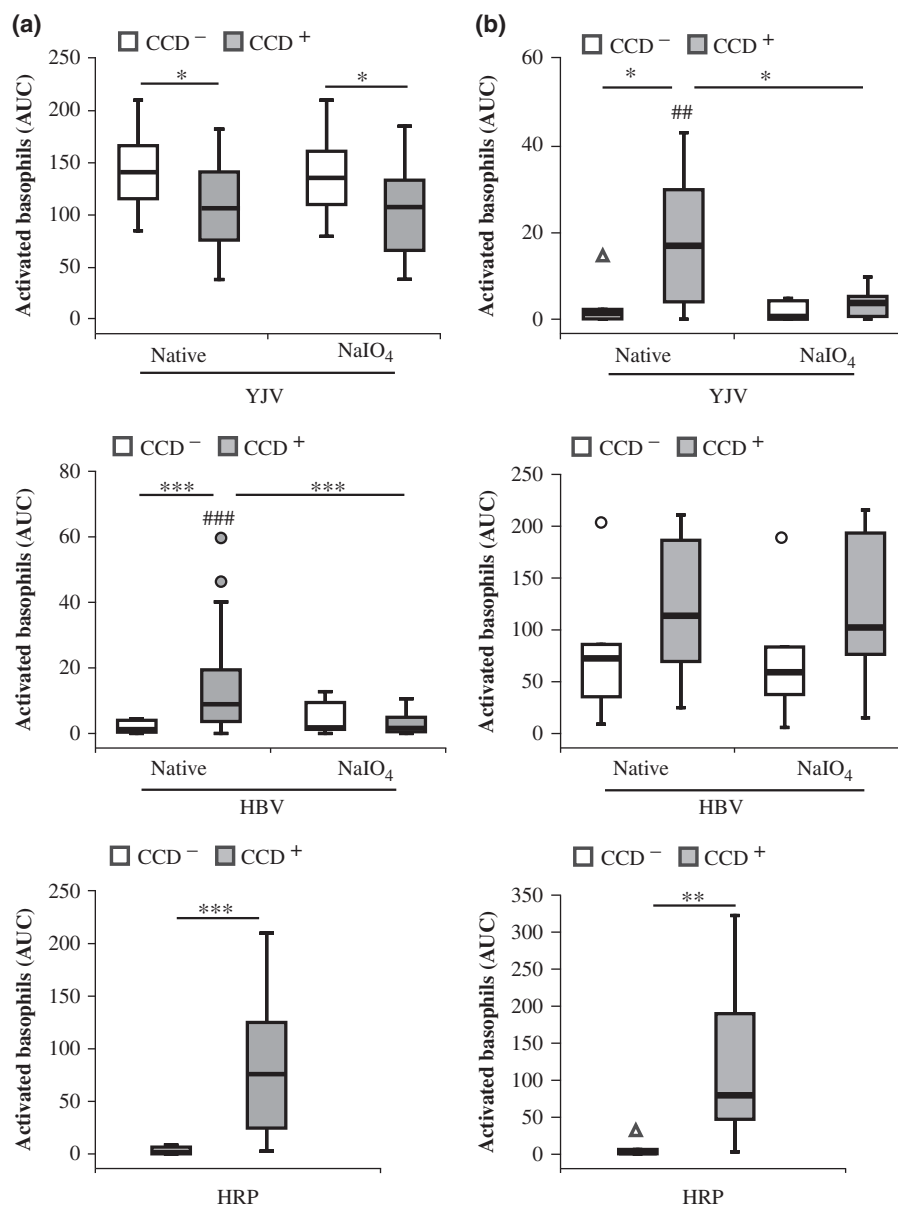


Fig. 4. Comparison of basophil activation in patients with a mono-positive intracutaneous skin test (IC). Results are given as area under the curve (AUC) for stimulation with native and NaIO₄-treated yellow jacket venom (YJV), native and NaIO₄-treated honeybee venom (HBV), and horseradish peroxidase (HRP). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (a) Patients with positive YJV-IC. Outliers: BW-83, BW-26. *** $P < 0.001$ between native YJV and native HBV in cross-reactive carbohydrate determinant (CCD)-positive patients. (b) Patients with positive HBV-IC. Outlier: BW-12. ** $P < 0.01$ between native YJV and native HBV in CCD-positive patients.

BAT in patients with double-positive IC identify a heterogeneous CCD-positive patient group

Because the group of skin-test double-sensitized patients comprises patients with primary sensitization to YJV as well as patients with primary sensitization to HBV and patients with independent sensitizations to both venoms, a simple classification into CCD-negative and CCD-positive patients for evaluation of BAT results was not feasible. Thus, analysis using AUC calculations was also not applicable.

All in all, five different patient subgroups were identified. A scheme of subgroups is shown in Fig. 6; examples for each subgroup are given in Fig. 7. Classification revealed one CCD-negative group (5/18): basophils of these patients showed a positive reaction upon stimulation with both hymenoptera venoms in their native as well as in their modified forms and no activation upon stimulation with HRP (i.e.: BW-25 and BW-45, Fig. 7a). Moreover, four different CCD-positive groups were identified (Fig. 6). Among them is one group without (2/13) and one with (3/13) basophil activation upon stimulation with

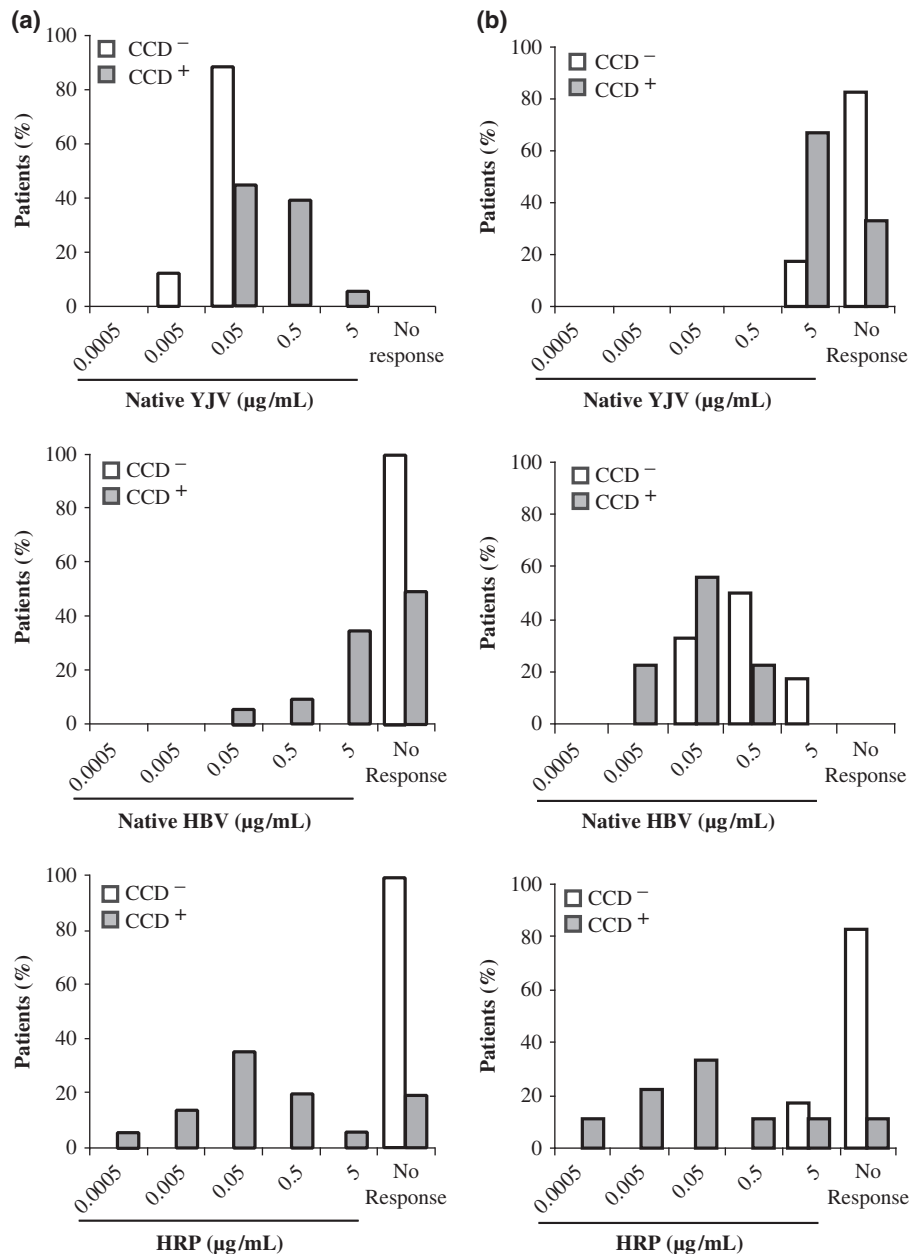


Fig. 5. Differences in the activation threshold of allergen-induced basophil activation in patients with a mono-positive intracutaneous skin test (IC). Presented are stimulations with native yellow jacket venom (YJV), native honeybee venom (HBV), and horseradish peroxidase (HRP). Bars indicate the lowest allergen concentration at which a basophil activation occurred. Stimulation was considered positive when the activation of basophils was more than 10% above the negative control. The total patients in one group are set to 100%. (a) Patients with positive YJV-IC, (b) patients with positive HBV-IC.

HRP, but no major differences between native and CCD-depleted YJV and HBV (i.e.: BW-98 and BW-80, Fig. 7b). Interestingly, the third and fourth CCD-positive group (7/13; 1/13) revealed results similar to those obtained in the CCD-positive skin-test mono-sensitized groups: The third group showed a strong reaction to both native and CCD-depleted YJV, while only the native but not the CCD-depleted HBV induced a significant activation of basophils (i.e.: BW-99, Fig. 7b). The fourth group showed the same results reciprocally (BW-101; Fig. 7b). HRP

induced an activation of basophils in all patients of the third and fourth patient groups.

Regarding the thresholds for activation with the different allergens in the BAT, those patients who distinguish between both forms of HBV showed an activation of basophils with native HBV mostly at 10–100 times higher concentrations compared with native YJV. Activation occurred mainly in the 0.5–5 µg/mL range (Fig. 7b and not shown). Interestingly, in CCD-negative patients and in those patients without differentiation between the native

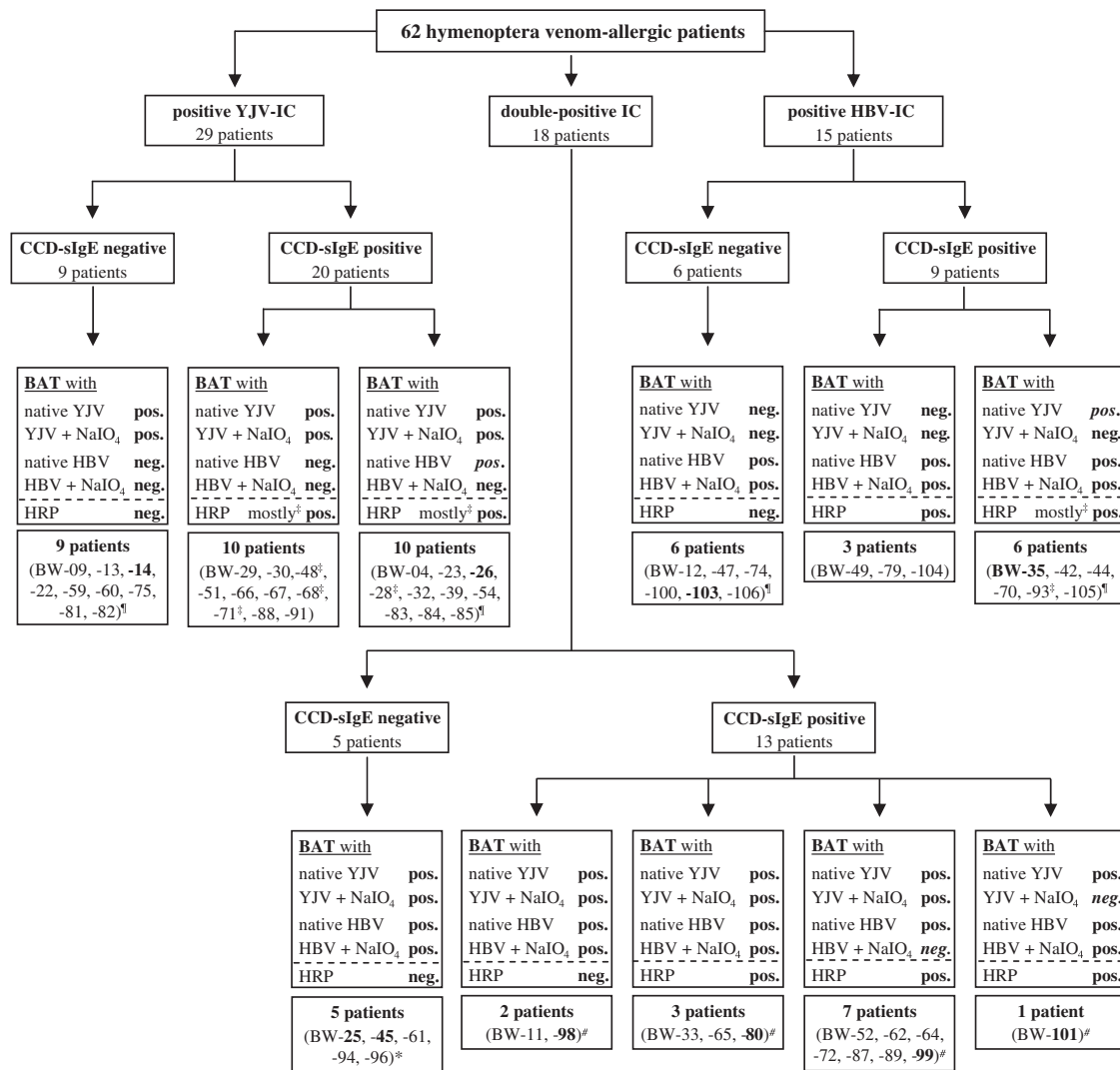


Fig. 6. Scheme of subgroups identified by intracutaneous skin test, specific IgE, and basophil activation test of all hymenoptera venom-allergic patients investigated. For further patient details, see Table 1. BAT, basophil activation test; IC, intracutaneous skin test; HBV, honeybee venom; HRP, horseradish peroxidase; neg., negative; pos.: positive; YJV, yellow jacket venom. [‡]Marked patients were negative with HRP in BAT analysis. [†]Patients highlighted in bold are exemplarily shown in Fig. 2 (BW-26) and 3. ^{*}Patients highlighted in bold are exemplarily shown in Fig. 7a. [#]Patients highlighted in bold are exemplarily shown in Fig. 7b. Results highlighted in italics are of special interest.

and the modified venoms, the threshold for activation between YJV and HBV also differed in some patients by up to two orders of magnitude (i.e.: BW-25, Fig. 7a).

Discussion

In the present study, 62 hymenoptera venom-allergic patients, classified according to their skin-test results with YJV and HBV, and the existence of CCD-sIgE (determined by CAP analyses), were investigated. In patients with only positive YJV-IC, CCD-negative as well as CCD-positive patients revealed no differences in the BAT when basophils were stimulated with either glycosylated or CCD-depleted YJV. The same was true, vice versa, for patients with only positive HBV-IC. Thus, in both mono-sensitized

groups, the outcome of the BAT was independent of the presence or absence of carbohydrate epitopes when applying the venom that was positive in the skin test. Besides, these results corroborate that the protein epitopes in the periodate-treated venoms had remained intact. In CCD-positive patients with mono-positive IC, stimulation with the venom that did not induce a positive skin reaction led to an activation of basophils in 50% or 67% of the patients, and this activity was lost upon periodate treatment. Similar observations were also found with one venom in 62% of CCD-positive patients with double-positive IC. Thus, in all three CCD-positive subgroups investigated, the loss of basophil activation can clearly be ascribed to the destruction of carbohydrate epitopes. The observation that glycoprotein-linked *N*-glycans are

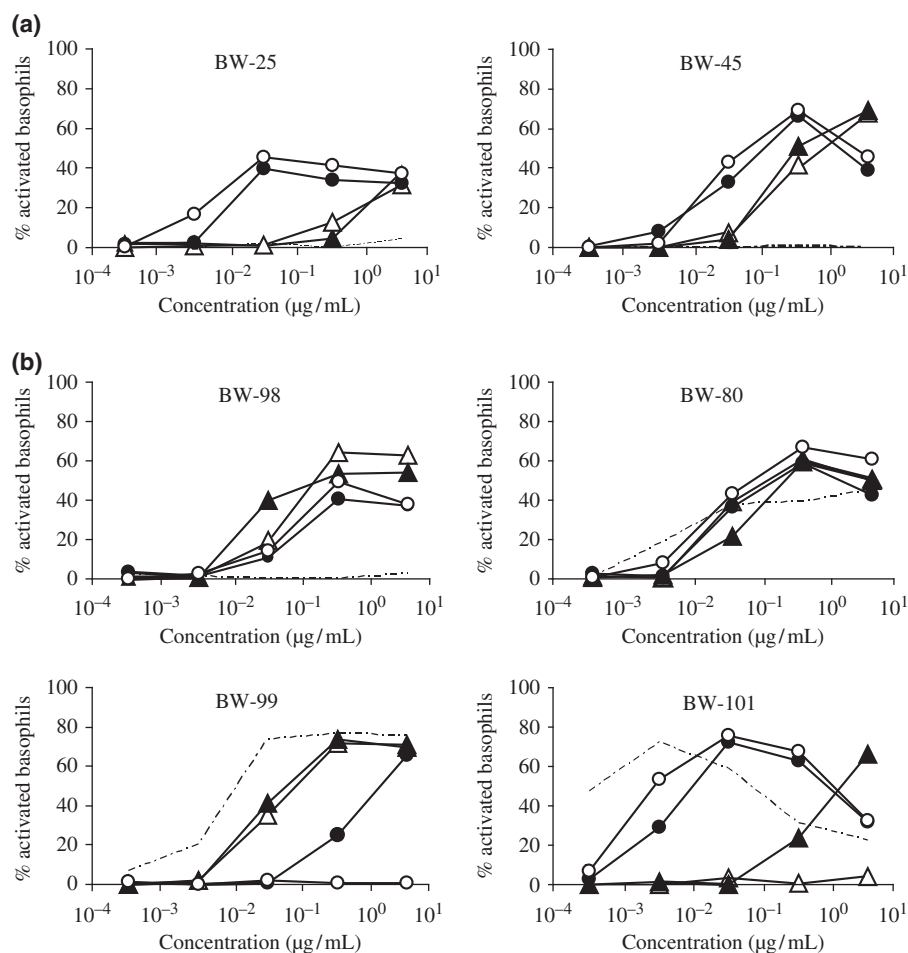


Fig. 7. Basophil activation in representative patients with a double-positive intracutaneous skin test. (a) Cross-reactive carbohydrate determinant (CCD)-negative patients. (b) CCD-positive patients. ▲, native yellow jacket venom; △, NaIO₄-treated yellow jacket venom; ●, native honeybee venom; ○, NaIO₄-treated honeybee venom; -, horseradish peroxidase.

able to induce biological activity in the BAT was further confirmed by the positive results obtained with HRP, an unrelated glycoprotein of plant origin. The strong activation detected with HRP in CCD-positive patients compared with the activation achieved with venom-CCD might be due to the fact that HRP carries at least seven *N*-glycan chains [33] and was applied as a purified plant glycoprotein. In contrast, HBV and YJV were used as raw extracts containing only some glycoproteins, namely phospholipase A2, hyaluronidase, acid phosphatase, and serine protease (HBV), or hyaluronidase (YJV), which carry one to four glycan chains [21]. We assume that the amount of activated basophils depends on the abundance of carbohydrate epitopes in the solution administered.

In earlier studies, results similar to those achieved here were obtained in histamine-release assays with native (glycosylated) vs. recombinant (non-glycosylated) plant and food allergens [12, 13, 15]. Because the patients investigated in these studies, in addition to the positive histamine-release assay with the glycosylated but a negative assay with the non-glycosylated allergen, displayed

sIgE predominantly against CCDs, and suffered from allergic symptoms, such studies support the hypothesis that carbohydrate structures may be clinically relevant. However, in the present study, most CCD-positive patients investigated (29/42) were skin-test mono-positive. Further, the activation of basophils by venom-CCD was induced only in 50–67% of the patients investigated and was significantly lower compared with activation by venom protein. Moreover, this activation occurred mainly when the venom was applied in high concentrations. In addition, it seems that the culprit venom was always recognized via protein-sIgE in the BAT. Thus, these results clearly point to a clinical irrelevance of CCD-sIgE in all patients analysed. Taking all this into account, it seems that CCD-based activation of basophils obscures activation by the true sensitizing venom. Therefore, the BAT performed with native, glycosylated hymenoptera venoms does not appear to be an appropriate tool to identify the culprit insect in patients with CCD-sIgE.

In the literature, it is shown that the BAT is a highly specific and sensitive test for diagnosing hymenoptera-

venom allergy [23–26, 28]. Because in most of the CCD-positive patients investigated here (29/42), the culprit insect could be identified by a mono-positive IC whereas the BAT performed with native venoms was double positive in 50–67% and thus failed to identify the culprit venom, its specificity and relevance for diagnosis is challenged by the present study. However, because all 16 non-allergic control individuals investigated were negative in the BAT and the activation of basophils with the venom that did not induce a positive IC can clearly be ascribed to the occurrence of sIgE antibodies against CCDs, the activation is highly specific. Moreover, this positive BAT response seems to be most likely a result of the BATs' high sensitivity. As shown in a recent report [29], it appears that the BAT has a higher sensitivity compared with IC and thus, this may also explain the discrepant BAT and IC findings in the majority of CCD-positive patients. In this context, it should also be mentioned that our data suggest that the BAT only reflects sIgE occurrence (but more sensitive than serum sIgE determination) and the ability of cross-linking sIgE molecules on the cell surface but is not able to differentiate between a clinically relevant or irrelevant sensitization, as it is discussed in the literature [34, 35]. It seems that the decision on elicitation of allergic symptoms is not made at the point of cross-linking sIgE-antibodies on the surface of basophils and mast cells but at a different point, e.g. on the level of regulatory T cells. However, irrespective of whether the BAT is able to distinguish between clinically relevant or not, due to the BATs' higher sensitivity, this test seems to be a reliable tool to detect hymenoptera venom sensitizations in patients with a history of allergic reactions but undetectable venom-sIgE and negative skin tests as, e.g. recently shown by Korosec *et al.* [29] and recommended in actual guidelines [27].

In conclusion, our data suggest that, irrespective of whether CCD-sIgE may have clinical relevance in certain patients, the BAT performed with native hymenoptera venoms does not offer an unambiguous final judgement to differentiate co-sensitization from cross-reactivity in hymenoptera venom-allergic patients. Additional inhibition tests as proposed by Jappe *et al.* [19] or sIgE assays with recombinant proteins expressed in *E. coli* (non-glycosylated) like phospholipase A2 for HBV and antigen 5 for YJV as proposed recently by Müller *et al.* [5] might be more reliable methods. Moreover, it is clear that the BAT is not able to differentiate between clinically relevant and irrelevant sensitizations at least in hymenoptera-venom allergy and involvement of CCD-sIgE. Because our data suggest in the majority of patients the clinical irrelevance of CCD-sIgE, and the double-positive IC in some patients may also be due to CCDs in one venom, the use of chemically treated proteins without CCD epitopes or a mixture of natively folded, recombinant allergens lacking only IgE-binding CCD epitopes (expression recently

shown for YJV- and HBV-hyaluronidases [36]) in BAT analyses should clearly improve their diagnostic value in the majority of CCD-positive patients.

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