

Identification by immunoblot of venom glycoproteins displaying immunoglobulin E-binding *N*-glycans as cross-reactive allergens in honeybee and yellow jacket venom

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Summary

Background IgE antibodies against carbohydrate epitopes have been identified recently as a major cause of *in vitro* double positivity to honeybee (HB) and vespid venom in patients with stinging-insect allergy. As these antibodies possibly have low clinical relevance they may be misleading in the diagnosis of venom allergy.

Objective To confirm the role of carbohydrate epitopes in double positivity and to locate the responsible glycoallergens in HB and yellow jacket (YJ) venom by western blot.

Methods Immunoblot inhibition using HB venom, YJ venom and two glycoprotein sources displaying 1-3-fucosylated *N*-glycans (i.e. oilseed rape (OSR) pollen, and the synthetic neo-glycoprotein fucosylated/xylosylated *N*-glycans from bromelain coupled to bovine serum albumin (MUXF-BSA)) as inhibitors were performed with sera from 15 double-positive patients with stinging-insect allergy. Additionally, reactivity with blotted hymenoptera venoms of a carbohydrate-specific rabbit antiserum against OSR pollen was investigated.

Results Major venom glycoallergens binding with carbohydrate-specific human IgE and rabbit IgG were detected in HB venom at 42 (hyaluronidase (HYA)), 46, 65 and 95 kDa, and in YJ venom at 38 and 43 kDa (HYA). Antibody binding to these allergens was completely lost after periodate treatment. Glycans of HB phospholipase were bound by patients' IgE only after protein denaturation. In 10 of the 15 patients the reactivity was with the second venom because of carbohydrates alone. The high-molecular-weight glycoallergens identified in HB venom probably correspond to similar proteins described earlier, including allergens B and C. The 38-kDa YJ allergen might represent a homologue of V mac 3.

Conclusions The data confirm the proposed role of carbohydrate-specific IgE in double positivity to HB and YJ venom and shed new light on some previously described minor hymenoptera allergens of uncertain clinical significance. The consideration of carbohydrate-specific IgE may allow to discriminate between patients with potentially relevant and patients with non-relevant double sensitization.

Keywords α -1-3-fucose, carbohydrate epitopes, CCDs, cross-reactive carbohydrate determinants, honeybee venom allergy, hymenoptera allergy, *N*-linked glycans, oilseed rape, venom double positivity, yellow jacket venom allergy

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Introduction

Simultaneous sensitization to honeybee (HB) and vespid venom is encountered in up to 50% of patients with suspect hymenoptera allergy undergoing routine allergy diagnosis [1–3]. Although only a minority of these patients reports adverse reactions from both insects [4, 5], double positivity remains a central diagnostic problem for the allergist. First, it raises the question about the potential clinical relevance of the test

results in the individual subject and, second, it often makes additional diagnostic measures necessary to identify the appropriate venom to be used for desensitization treatment [3, 6, 7]. Reciprocal RAST inhibition with HB and vespid venom is the method of choice to discriminate between patients with true double sensitization to both insects and those with primary sensitization to one venom and cross-sensitization to the other [6–8]. HB and wasp venom hyaluronidases (HYAs), which have about 50% sequence identity [9, 10], are considered to be the allergens responsible for most of this cross-reactivity [11, 12].

We recently extended conventional reciprocal RAST inhibition by including also naturally occurring, as well as synthetic, glycoproteins as inhibitor proteins and thereby could identify oligosaccharide side chains of venom glycoproteins as

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another important and thus far unrecognized immunogenic structure responsible for cross-sensitization to HB and yellow jacket (YJ) [13]. According to this data, more than half of all double-positive patients have IgE antibodies directed against venom glycans, and in a substantial percentage of these patients the reactivity with the second venom is entirely because of carbohydrate. This finding is important, because IgE antibodies against carbohydrate epitopes may have low, if any, clinical relevance [14]. If this holds true also for stinging-insect allergy, many of the positive RAST results encountered in double-positive patients have to be considered 'false-positive' and misleading.

The glycan structures involved in venom double positivity are *N*-glycans bearing a fucose residue α -1-3-linked to the glycan core [13]. These glycans are increasingly recognized as cross-reactive carbohydrate determinants (CCDs) mediating extensive cross-reactivity between otherwise unrelated animal and plant glycoproteins [15–18]. As for hymenoptera venoms, there is evidence for the occurrence of fucosylated *N*-glycans in HB phospholipase A₂ (PLA₂), acid phosphatase (AP) and HYA [19–21], but specific binding of patients' IgE with venom glycans has been demonstrated only for PLA [22, 23]. No comparable glycosylation data exist for vespid venoms, and the allergens binding with glycan-specific IgE of human sera are not known.

The aim of the present study was to locate the allergens in HB and YJ venom specifically binding with carbohydrate-specific IgE, and to confirm by western blot and western blot inhibition our previous findings on the role of venom carbohydrates in multiple hymenoptera sensitivity.

Patients and methods

Patients

Fifteen patients with a history of a systemic or severe local reaction after a hymenoptera sting, and with a positive RAST

(Pharmacia CAP-FEIA, Pharmacia, Sweden) of at least class 3 to both HB and YJ venom were enrolled in the study. Demographic data, RAST results, total IgE, and results from venom skin prick testing (maximum dose 300 µg/mL) are summarized in Table 1. Thirteen of the patients (pts 1–13) had also a positive RAST to oilseed rape (OSR) pollen. Because OSR pollen is rich in fucosylated *N*-glycans, a positive RAST result was considered to indicate the presence of carbohydrate-specific IgE in a particular serum [13]. On the basis of history and reactivity with OSR pollen, patients were grouped as follows:

Group 1 (pts 1–10): Ten patients with a history of vespid allergy (9 × YJ, 1 × European hornet) and with a positive RAST to OSR pollen. We hypothesize that the IgE in these sera specifically binding with HB venom is directed against HB venom carbohydrates.

Group 2 (pts 11–13): Three patients with a history of HB allergy and with a positive RAST to OSR pollen. We hypothesize that the IgE in these sera specifically binding with vespid venom is directed against vespid venom carbohydrates.

Group 3 (pts 14–15): Two patients with a history of vespid allergy and with a negative RAST to OSR pollen. We hypothesize that IgE binding to HB venom in these sera is not directed against HB venom carbohydrates but is either because of cross-reactivity with HB HYA or because of true double sensitization.

Hymenoptera venoms

Lyophilized pure HB venom and YJ venom sac extracts (mixture of *Vespula vulgaris* and *germanica*) were purchased from Vespa (Vespa Laboratories, Spring Mills, PA, USA).

Glycoproteins displaying fucosylated *N*-glycans used in inhibition experiments

Two glycoprotein sources containing complex *N*-glycans were used in the inhibition studies, i.e. (1) extract from OSR

Table 1. Anamnestic data, total IgE, RAST results, and results from skin prick testing (SPT) of the 15 patients studied

Pt.	Age (years)	Sex	Culprit insect	Clinical reaction	Total IgE	RAST HB	RAST YJ	RAST OSR	SPT (HB/YJ)	Remarks
1	52	f	YJ	II	1360	4.3	3.7	4.1	100/100	Pollinosis
2	37	m	YJ	II	120	3.9	3.0	3.9	300?/100	
3	37	m	YJ	I	1030	4.1	5.7	4.0	n.d.	
4	38	m	YJ?	I	430	4.4	3.0	4.6	300?/100	Pollinosis
5	20	m	YJ?	II	120	3.0	3.7	3.8	Neg/100	
6	55	f	YJ	III	580	3.2	4.6	3.9	Neg/100	
7	33	f	YJ	LLR	90	3.2	4.0	3.5	Neg/100	Pollinosis
8	48	m	YJ	III	80	3.2	3.6	3.3	Neg/300	
9	61	f	HO	IV	780	3.9	4.0	4.0	n.d.	
10	12	m	YJ?	I	2000	5.3	3.9	4.8	100/100	Pollinosis
11	8	m	HB	III	1850	5.1	3.5	4.1	300/neg	Pollinosis
12	23	f	HB	IV	1840	5.7	5.1	5.3	Neg/10	
13	42	f	HB	LLR	360	4.3	4.0	4.6	100/100	Pollinosis
14	74	f	YJ	IV	140	3.8	3.5	neg	n.d.	
15	13	m	YJ?	II	1320	3.9	3.8	neg	100/100	

All patients had a RAST of at least class 3 to both venoms. Patients 1–13 (pts 1–13) had also a positive RAST to OSR pollen suggestive of carbohydrate-specific IgE in the respective sera. Culprit insect refers to the insect suspected by the patient. Severity of clinical reactions is given in terms of the Mueller grade. RAST is given in term of RAST class. Figures for SPT refer to the allergen concentration (µg/mL) eliciting a weal of at least 3 mm. m, male; f, female; LLR, large local reaction; HB, honeybee; YJ, yellow jacket; HO, European hornet (*Vespa crabro*); OSR: oilseed rape pollen; neg, negative; n.d., not done.

(*Brassica napus*) pollen, and (2) the neo-glycoprotein fucosylated/xylosylated *N*-glycans from bromelain coupled to bovine serum albumin (MUXF-BSA). Pollen from OSR was purchased from Kabi-Pharmacia-Allergon (Ängelholm, Sweden) and extracts prepared as described earlier [24]. High-molecular-weight (HMW) glycoproteins have been found to be major IgE-binding components in OSR pollen [24], and analysis of their *N*-glycans indicated the presence of many glycans with 1-3-linked fucose [13]. Different from other pollens, OSR pollen contains also considerable amounts of MMF glycans with core 1-3 fucosylation and two terminal mannoses (Man α 6(Man α 3)Man β 4GlcNAc β 4(Fuc α 3)GlcNAc β Asn), identical to some glycans present in HB PLA₂ and HYA [20, 21].

MUXF-BSA is a synthetic neo-glycoprotein containing a core α 1,3-fucosylated and xylosylated *N*-glycan (Man α 6-Man β 4(Xyl β 2)GlcNAc β 4(Fuc α 3)GlcNAc β -Asn) from pineapple stem bromelain. Preparation of the neo-glycoprotein is described in detail in reference [17]. In brief, commercially available bromelain (Sigma) was digested with pepsin in 5% formic acid to obtain small MUXF glycopeptides which were then cross-linked to bovine serum albumin (BSA). According to amino sugar analysis the resulting neo-glycoprotein had about 3–4 mol glycopeptide per mol protein.

Rabbit anti-carbohydrate antiserum

Glycoproteins in HB and YJ venom displaying CCDs were identified by means of Bra n, a polyclonal rabbit antiserum raised against crude OSR pollen extracts. This serum binds strongly with extracts from rubber latex, many pollens, nuts and fruits as well as with *Ficus benjamina* [25], and has been shown previously to compete successfully with carbohydrate-specific IgE for antibody binding to HB and YJ venom glycans [13].

Gel electrophoresis, western blotting

HB and YJ venom were separated by SDS-PAGE using 13.5% homogenous gels (10.5 $\times$ 8.0 $\times$ 0.1 cm) with 5% stacking gels at 25 mA and constant voltage. One hundred and twenty grams of lyophilized venom protein (corresponding to approximately 6 μ g/lane) were dissolved in sample buffer (Laemmli buffer without mercaptoethanol) [26] and immediately loaded onto the gels without further protein denaturation or protein reduction. To compare the electrophoretic migration and allergenicity of native venom proteins with that of reduced and denatured proteins, SDS-PAGE was also carried out with venom reduced and denatured by heating the extract to 95 °C for 5 min in the presence of 5% mercaptoethanol.

Electrophoretically separated proteins were blotted onto nitrocellulose according to Towbin [27]. After transfer, sheets were cut into strips of 3 mm and blocked with BSA-containing buffer (50 mmol/L sodium phosphate, 0.5% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin and 0.05% (w/v) sodium azide, giving a final pH of 7.5) for 30 min. Each strip was incubated overnight with 1 mL of serum (diluted either 1 : 10 or 1 : 5) at 4 °C under continuous shaking, washed twice in buffer for 30 min and incubated overnight at room temperature with ¹²⁵I-labelled rabbit anti-human IgE (Phar-

macia, Sweden) diluted to achieve 200 000 c.p.m. per strip. Strips were washed again, dried at room temperature and exposed to a high-performance autoradiography film (Hyperfilm MP, Amersham, England) at –70 °C for 3–5 days. Rabbit IgG was detected by a 1 : 2000 diluted ¹²⁵I-labelled donkey anti-rabbit antiserum (Amersham, Buckinghamshire, UK).

Immunoblot inhibition

For immunoblot inhibition, the patient's serum was mixed with the respective inhibitor protein to achieve a final protein concentration of 30 μ g/mL. BSA was used as a negative control. After overnight co-incubation at 4 °C, immunoblotting was performed as usual.

Periodate treatment

To study the effect of periodate treatment on IgE binding to venom proteins, single nitrocellulose strips with electroblotted allergens were treated for 1 h with periodate (10 mM periodic acid in 0.1 M sodium acetate buffer, pH 5.5, 20 °C) to oxidize glycoprotein oligosaccharides. Subsequently, the IgE-binding patterns were compared with those from untreated strips.

Identification of venom allergens by matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry

Coomassie blue-stained bands of electrophoretically separated venom proteins were excised, destained and carboxymethylated as described in reference [28]. For tryptic in-gel-digest and peptide extraction another protocol was followed [29]. The tryptic peptides were analysed by MALDI–TOF mass spectrometry with a Waters Micromass Q-TOF Ultima Global in positive ion mode. A *m/z* mass range from 500 to 4000 was scanned.

Results

Immunoblot IgE binding to venom allergens in patients from group 1 (pts 1–10) (Fig. 1)

The IgE from pts 1–10 consistently bound in HB venom to a 42 kDa band and another three well-delineated HMW bands of 46, 65 and approximately 95 kDa (Fig. 1). Additional blurred antibody binding occurred between 35 and 40 kDa. Only one patient (pt 10) reacted with HB PLA₂ (Fig. 1). Peptide mapping by MALDI–TOF mass spectrometry identified the 42-kDa band as HB HYA (Table 2). The band also contained some acid phosphatase as a few of the tryptic peptides were consistent with the partially known amino acid sequence of acid phosphatase [30] (L. Soldatova, personal communication). None of the peptides obtained from the HMW bands could be assigned to any of the known HB venom allergens, including Api m 7 (accession no. AY127579).

IgE binding to 46, 65 and 95 kDa was always completely inhibited by OSR pollen and by MUXF-BSA (Fig. 1, lanes OSR and MUXF, respectively) and was totally abolished after periodate treatment (Fig. 1, lanes PI) indicating that all

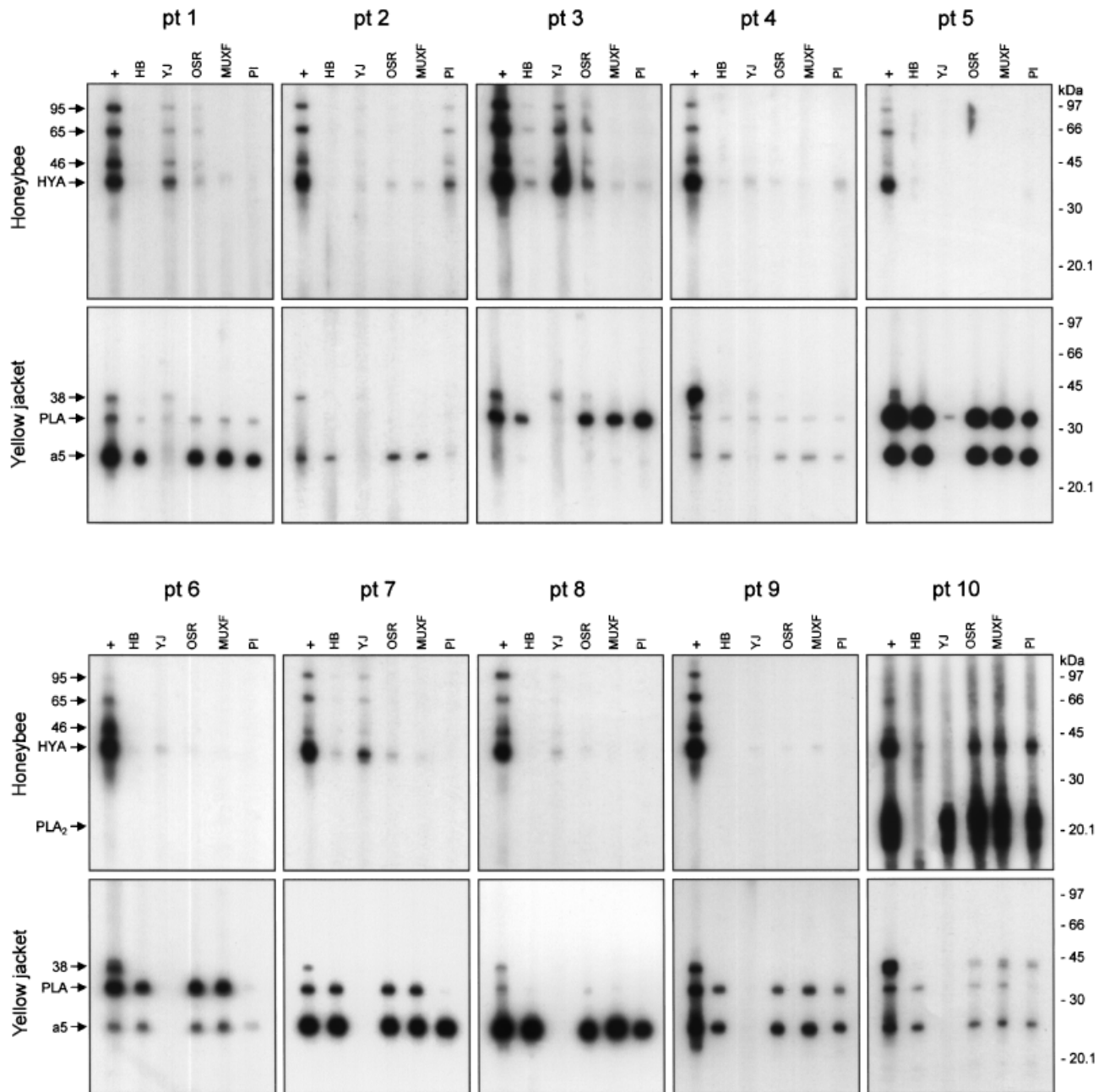


Fig. 1. IgE binding to hymenoptera venoms separated by non-reducing SDS-PAGE in 10 patients with a history of vespid allergy and a double-positive RAST to honeybee (HB) and yellow jacket (YJ) venom (patients (pts) 1–10). All patients had a positive RAST to oilseed rape pollen suggesting the presence of carbohydrate-specific serum IgE. Inhibition of antibody binding was performed by serum pre-incubation with 30 µg/mL of HB venom, YJ venom, oilseed rape (OSR) pollen extracts, and the neo-glycoprotein MUXF-BSA (MUXF). +, non-inhibited positive control; PI, IgE binding after periodate oxidation of carbohydrate side chains; HYA, hyaluronidase; PLA, phospholipase; a5, antigen 5. For history of patients see Table 1.

IgE was directed to carbohydrates. There was also substantial inhibition of IgE binding to these allergens by YJ venom (Fig. 1, lanes YJ). IgE binding to 42-kDa HB HYA was completely inhibited by OSR pollen and by MUXF-BSA and was totally abolished after periodate treatment in pts 1–9 indicating specific binding to carbohydrates, whereas it was only partially inhibited in pt 10. In the latter patient, antibody binding to HB HYA was completely inhibited by HB and by YJ venom indicating IgE binding to HYA carbohydrates as well as to common peptide epitopes in HB and YJ HYA.

Binding to HB PLA₂ in pt 10 was inhibited by HB venom alone suggesting specific binding with the PLA₂ peptide backbone. No inhibition was obtained ever with BSA (not shown).

In YJ venom, one serum (pt 2) reacted with a 24-kDa band (identified as antigen 5 by peptide mapping, see Table 2), one serum (pt 3) reacted with 32 kDa (identified as YJ PLA, see Table 2), and the remaining eight sera reacted with both allergens (Fig. 1). Furthermore, all sera bound with a 38-kDa band sometimes appearing as a doublet. Mass spectrometry

Table 2. Identification of honeybee and *Vespula* venom allergens by MALDI-TOF MS

Experimental mass [MH] ⁺	Position	Peptide sequence
<i>Apis mellifera</i> hyaluronidase (Q08169) 45% sequence coverage		
1971.93 (CAM), 1987.92 (CAM+MSO)	42–56	EFNVYWNVPTFMCHK
1283.63 (MSO)	68–77	YGILQNWMDK
799.47	119–124	HLQVFR
1192.66	125–134	DHLINQIPDK
2109.13	135–152	SFPGVGVIDFESWRPIFR
1234.65	153–162	QNWASLQPYK
1229.57	172–180	EHPFWDDQR
1358.72, 1374.69 (MSO)	192–202	YGQLFMEETLK
2184.14, 2200.15 (MSO)	244–261	MSWLFESDVLPSVYLR
2000.89 (<i>N</i> -glycan Man ₃ GlcNAc ₂ Fuc ₁), 2146.99 (<i>N</i> -glycan Man ₃ GlcNAc ₂ Fuc ₂)	262–269	WNLTSGER
657.41	270–276	VGLVGGR
1131.62	294–301	VLPYYWYK
706.35	307–312	DTDLSR
888.50	313–320	ADLEATLR
2238.14	322–342	ITDLGADGFIWGSSDDINT
723.37 (CAM), 737.39 (PAM)	345–349	CLQFR
<i>Vespula vulgaris</i> antigen 5 (Q05110) 44% sequence coverage		
817.38	70–75	EHNDFR
962.51	86–95	GNPGPQPPAK
3310.50 (2x CAM), 3324.59 (CAM+ PAM)	99–125	NLVWNDELAYVAQVWANQCQYGHDTCR
1607.84	130–145	YQVGQNVALTGSTAAK
927.47	169–176	FSGNDFLK
1536.74, 1552.74 (MSO)	177–189	TGHYTMVWANTK
2680.20 (CAM+MSO), 2694.21 (PAM+ MSO)	206–227	HYLVLCNYGPSGNFMNEELYQTK
<i>Vespula germanica</i> antigen 5 (P35784) 36% sequence coverage		
817.38	47–52	EHNDFR
962.51	63–72	GNPGPQPPAK
1576.81	107–122	YPVGQNVALTGSTAAK
998.51	146–153	FSENNFLK
1548.79, 1564.77 (MSO)	154–166	IGHYTMVWANTK
2590.17 (CAM), 2604.11 (PAM)	183–204	HYLVLCNYGPSGNFGNEELYQTK
<i>V. vulgaris</i> phospholipase (P49369) 50% sequence coverage		
1751.85 (CAM)	40–54	CPFNSDTVSIIEETR
1846.95	60–74	DLYTLQTLQNHPEFK
2951.63	77–103	TITRPVVFITHGFTSSASETNFINLAK
1117.57	132–140	YLYYPTAAR
1334.78	144–155	LVGQYIATITQK
1522.77	169–184	LIGHSLGAHASGFAGK
2550.20 (CAM)	194–215	YSEIIGLDPARPSFDSNHCSER
788.36 (CAM), 802.39 (PAM)	251–257	NQPGCGR
1255.58 (CAM), 1269.58 (PAM)	258–267	FFSEVCSHSR
1197.59 (CAM+MSO)	268–277	AVIYMAECIK
1226.63 (2x CAM)	278–287	HECCLIGIPK
1916.90 (CAM), 1930.91 (PAM)	316–332	GSFYVPVESTAPFCNNK
<i>V. vulgaris</i> hyaluronidase (P49370) 18% sequence coverage		
1894.07	47–63	IAIFYDPGEFPALLSLK
874.52	111–116	WRPIFR
2180.18, 2196.18 (MSO)	208–225	MSWLFNNQNVLLPSVYVR
986.51	226–233	QELTPDQR
742.46	234–240	IGLVQGR
688.43	247–252	ISNNLK

The experimental masses of all peptides deviated less than 25 p.p.m. from the calculated values. Peptides 262–269 from honeybee hyaluronidase was found *N*-glycosylated [28]. Other modifications are artefacts from sample preparation. CAM, carboxyamidomethyl-modified cysteine; PAM, propionamide-modified cysteine; MSO, methionine sulfoxide; *N*-glycan, *N*-glycan attached to peptide.

revealed traces of YJ HYA (SwissProt Acc. Nr. P49370) in this band but the majority of the tryptic peptides derived either from a different isoform of HYA or from an unknown

protein (see below). IgE binding to the 38-kDa allergen was always completely inhibited by HB venom, OSR pollen and by MUXF-BSA, and was totally abolished after periodate

treatment indicating specific reactivity with carbohydrates. IgE binding to YJ PLA and antigen 5 was totally inhibited by YJ venom but not or only weakly by HB venom, OSR pollen and MUXF-BSA, indicating specific binding with peptide epitopes.

Our hypothesis that cross-sensitization to HB venom in the vespid-allergic pts 1–10 involves carbohydrate epitopes was confirmed by western blot in all patients. In nine of them, the cross-reactivity with HB venom was because of carbohydrate alone and may be regarded as clinically non-relevant. Pt 10 appears to be truly double sensitized, as the IgE specifically bound to distinct peptide epitopes in HB and *Vespula* venom.

Immunoblot IgE binding to venom allergens in patients from group 2 (pts 11–13) (Fig. 2)

The IgE from pts 11–13 consistently bound in HB venom to HB HYA (42 kDa) and the same HMW proteins as did the IgE from pts 1 to 10 (Fig. 2). The serum from pt 11 reacted also with HB PLA₂. IgE binding to the HMW allergens was completely inhibited in all sera by OSR pollen and by MUXF-BSA (Fig. 2, lanes OSR and MUXF, respectively) and was totally abolished after periodate treatment (Fig. 2, lanes PI) indicating IgE binding to carbohydrates. Binding to HB HYA was not or not considerably inhibited by OSR pollen and MUXF-BSA, and was not abrogated after periodate treatment, indicating specific reactivity with peptide epitopes. The HB HYA-specific IgE of pt 13 cross-reacted with *Vespula* HYA.

All patients had IgE to the YJ 38-kDa allergen. The serum from pts 12 and 13 reacted also with YJ PLA and antigen 5. As in pts 1–10, IgE binding to the 38-kDa glycoallergen was always completely inhibited by HB venom, OSR pollen and by MUXF-BSA, and was totally abolished after periodate

treatment indicating specific binding with carbohydrates. IgE binding to YJ PLA and antigen 5 was only inhibited by YJ venom but not by the other inhibitors.

Our hypothesis that cross-sensitization to *Vespula* venom in the HB venom-allergic pts 11–13 involves carbohydrate epitopes was confirmed by immunoblot in all patients. In pt 11, the cross-reactivity with YJ venom was because of carbohydrate alone and may be regarded as clinically non-relevant. Pt 12 appears to be truly double-sensitized, as the IgE specifically bound to distinct peptide epitopes in HB and *Vespula* venom. Pt 13 appears to have primary vespid venom allergy with cross-sensitization to HB venom HYA.

Immunoblot IgE binding to venom allergens in patients from group 3 (pts 14–15) (Fig. 2)

The sera from pts 14 and 15 bound in HB venom with HYA only. IgE binding to HB HYA was directed against peptide epitopes as it was resistant to periodate treatment and was not inhibited by OSR pollen and MUXF-BSA. In YJ venom, both sera bound with *Vespula*-specific peptide epitopes on YJ PLA and antigen 5. In consistency with the finding of a negative RAST to OSR pollen in pts 14 and 15, the HMW HB venom allergens and the 38-kDa *Vespula* glycoallergen were not recognized. The data suggest that both patients were primarily sensitized to YJ venom and that some of their IgE cross-reacts with HB HYA.

Comparison of antibody binding to venom allergens after reducing and non-reducing SDS-PAGE (Figs 3 and 4)

Because we could not detect in the non-reduced YJ venom IgE binding to a 40–45 kDa allergen corresponding to YJ HYA, we compared the electrophoretic migration and IgE

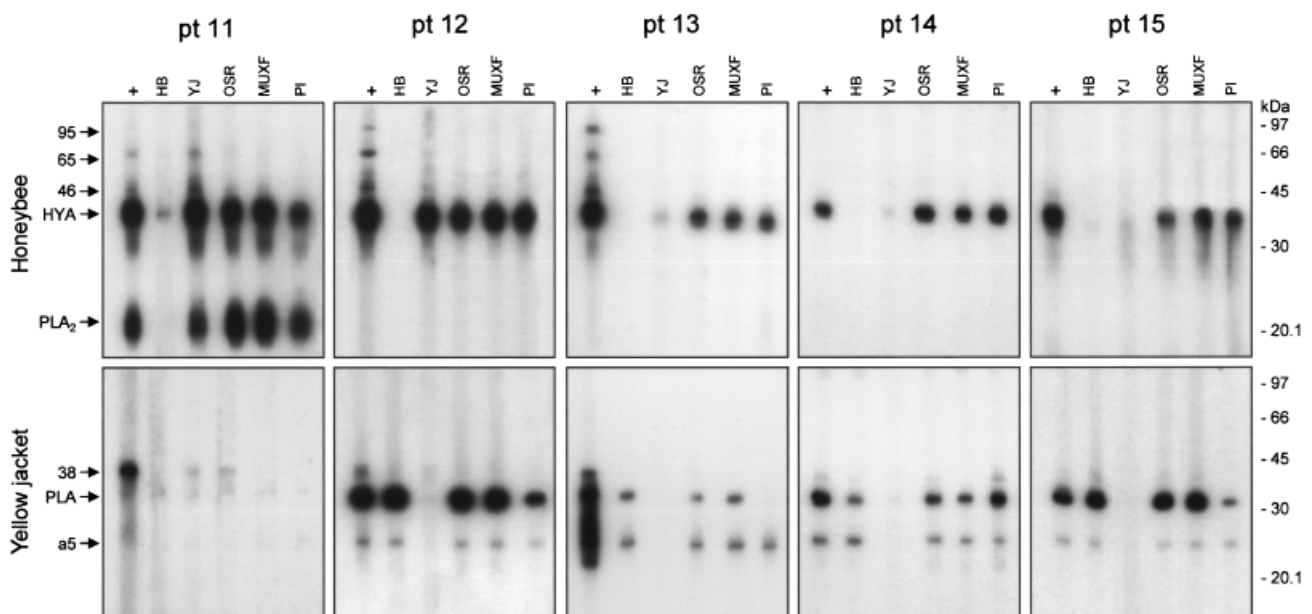


Fig. 2. IgE binding to hymenoptera venoms separated by non-reducing SDS-PAGE in three patients with a history of honeybee (HB) venom allergy and a double-positive RAST to HB and yellow jacket (YJ) venom (patients (pts) 11–13), and in two double-positive patients with vespid venom allergy (pts 14–15). Pts 11–13 had a positive RAST to oilseed rape pollen suggesting the presence of carbohydrate-specific serum IgE. RAST to oilseed rape (OSR) pollen was negative in pts 14 and 15 indicating the absence of carbohydrate-specific IgE. Inhibition of antibody binding was performed by serum pre-incubation with 30 µg/mL of HB venom, YJ venom, OSR pollen extracts, and the neo-glycoprotein MUXF-BSA (MUXF). +, non-inhibited positive control; PI, IgE binding after periodate oxidation of carbohydrate side chains; HYA, hyaluronidase; PLA, phospholipase; a5, antigen 5. For history of patients see Table 1.

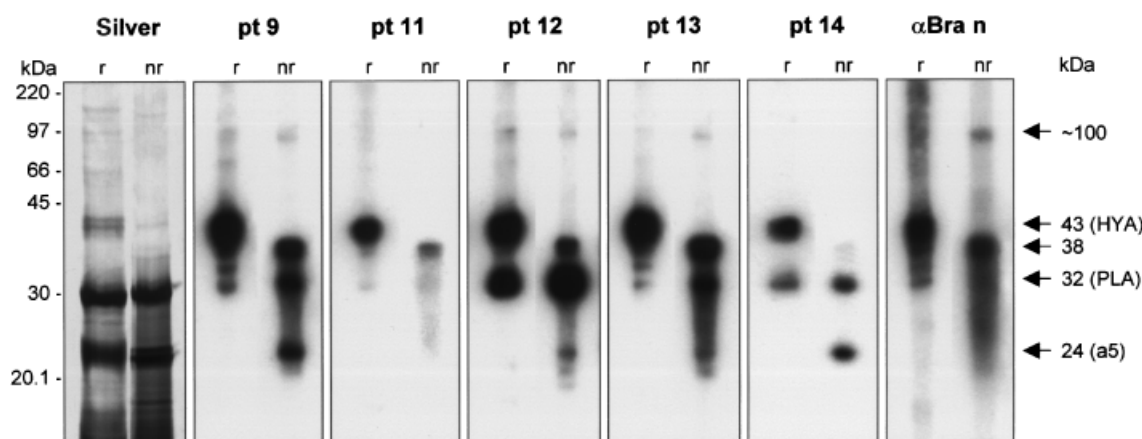


Fig. 3. Comparison of silver staining and antibody-binding patterns between reduced/denatured (r) and non-reduced (nr) yellow jacket (YJ) venom. Proteins were either reduced and denatured by boiling in the presence of mercaptoethanol before electrophoresis or immediately separated by SDS-PAGE without further treatment, and subsequently tested with sera from patients (pts) 9, 11–14, and with rabbit α Bra n, a carbohydrate-specific polyclonal rabbit antiserum raised against a crude oilseed rape pollen extract. IgE binding to 43 kDa YJ HYA was only seen with reduced venom (r lanes) whereas binding with antigen 5 was confined to the non-reduced venom (nr lanes). HYA, hyaluronidase; PLA, phospholipase; a5, antigen 5.

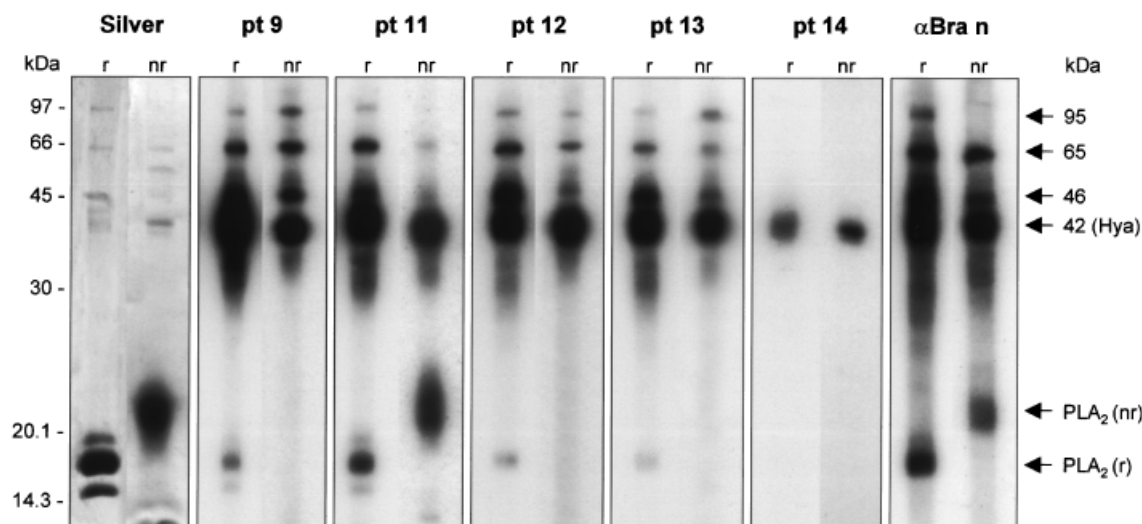


Fig. 4. Comparison of silver staining and antibody-binding patterns between reduced/denatured (r) and non-reduced (nr) honeybee (HB) venom. Proteins were either reduced and denatured by boiling in the presence of mercaptoethanol before electrophoresis or immediately separated by SDS-PAGE without further treatment, and tested with sera from patients (pts) 9, 11–14, and with α Bra n, a carbohydrate-specific polyclonal rabbit antiserum raised against a crude oilseed rape pollen extract. HB PLA₂ migrated differently under reducing and non-reducing conditions. Carbohydrates of PLA₂ were bound by patients' IgE only after protein denaturation (pts 9, 12 and 13). HYA, hyaluronidase; PLA, phospholipase; a5, antigen 5.

binding capacity of hymenoptera venom allergens under reducing and non-reducing conditions.

A 43-kDa double band in the reduced *Vespa* venom could be identified as HYA (P49370) by MALDI-TOF mass spectrometry (Table 2), consistent with the allergen's expected size [9], whereas in non-reduced extracts only faint bands around 38 kDa were visible (Fig. 3, protein silver staining). Some minor tryptic peptides obtained from the 38-kDa protein corresponded to YJ HYA, indicating co-migration of small amounts of HYA within this band; however, the majority of peptides derived either from a different isoform of YJ HYA or from an unknown protein. The same pattern of unidentified peptides was also found in the 43-kDa YJ HYA band. YJ PLA and antigen 5 did not migrate differently under reducing and non-reducing conditions.

In the corresponding immunoblots performed with selected patients' sera, antibody binding to the reduced venom was strongest at 43 kDa (YJ HYA) along with additional binding at 32 (YJ PLA), 34 and 38 kDa (Fig. 3, pts 9 and 11–14, r lanes). The same proteins were bound by rabbit anti-Bra n (Fig. 3, α Bra n, r lane) identifying all proteins as glycoproteins. In the non-reduced YJ venom, human sera as well as anti-Bra n strongly bound with a 38-kDa band but no binding at 43 kDa was observed (Fig. 3, nr lanes). Weak binding around 100 kDa was seen with all carbohydrate-specific sera in either venom preparation (pts 9, 11–13, anti-Bra n). In all, the data suggest that vespine HYA did not migrate substantially into the gel under non-reducing conditions. On the other hand, under reducing conditions, antigen 5 was not bound by any of the sera, and reactivity with

reduced YJ PLA was weaker than reactivity with the non-reduced allergen.

HB PLA₂ migrated under non-reducing conditions as a band of higher molecular weight (20.5–22 kDa) as compared with the reduced allergen (15.5/18/20 kDa triplet) (Fig. 4). Interestingly, carbohydrate-specific IgE bound only with denatured HB PLA₂ but not with the native protein (Fig. 4; pts 9, 12, 13), whereas the PLA₂ peptide-specific IgE of pt 11 reacted with either venom preparation. Rabbit anti-Bra n strongly bound with HB HYA and all HMW allergens as well as with PLA₂ in reduced and unreduced venom (Fig. 4; Bra n).

Impact of periodate treatment on antibody binding to YJ antigen 5 and PLA

Apart from the strong reduction of IgE binding to venom carbohydrate epitopes, periodate treatment also decreased IgE binding to YJ PLA and antigen 5 obviously not related to carbohydrate. In six out of 13 sera reacting with YJ PLA, antibody binding was reduced or even completely extinguished by periodate while it was not or not considerably inhibited by OSR pollen and MUXF-BSA (Figs 1 and 2; pts 6, 7, 10, 12, 13, 15; PI lanes). IgE binding to antigen 5, which is not a glycoprotein at all, was reduced by periodate in two out of 13 patients (Fig. 1, pts 2 and 6).

Discussion

This study in patients with stinging-insect allergy and double-positive RAST to HB and YJ venom shows that crude OSR pollen extracts and the synthetic neo-glycoprotein MUXF-BSA, both displaying 1-3-fucosylated *N*-glycans, can inhibit IgE binding to certain venom allergens in the western blot, providing further evidence for the involvement of carbohydrate-specific IgE in HB and YJ venom double positivity.

In consistency with our previous findings from RAST inhibition [13], the cross-reactivity with the second venom was found to be entirely because of carbohydrate epitopes in many patients. As long as there is no clear evidence for a clinical role of anti-carbohydrate IgE in venom allergy such cross-reactivity may be regarded as clinically less important. The results from skin prick testing showing that most patients with IgE to HB venom carbohydrates do not have a positive skin test to HB venom would agree with the concept of poor biologic activity of anti-carbohydrate IgE, although this needs to be confirmed by intradermal testing in a larger sample. Because conventional reciprocal RAST inhibition does not disclose whether cross-sensitization is mediated by peptide or by carbohydrate epitopes, inclusion of fucosylated glycoproteins in inhibition experiments should be helpful to discriminate between patients with potentially relevant and patients with presumably irrelevant co-sensitization. As an alternative, a simple RAST to OSR pollen or any other adequate glycoprotein source (e.g. bromelain) would give a clue on the potential involvement of carbohydrate-specific IgE. It should be kept in mind, however, that the presence of carbohydrate-specific IgE in a particular serum does not exclude the concurrent existence of peptide-specific IgE to both venoms, as encountered in pts 10, 12 and 13 in this study. Furthermore, reactivity with OSR pollen may be occasionally

because of pollen panallergens such as profilin and Ca-binding proteins but not because of carbohydrates [24]. Bromelain, on the other hand, has the disadvantage of a narrow glycan repertoire not depicting all carbohydrate determinants found in hymenoptera venoms. Taking into account that carbohydrate-specific IgE may also interfere with the proper diagnosis of pollen allergy, food allergy and rubber latex allergy, the development of commercial RAST assays utilizing well-characterized synthetic glycoproteins would be welcome.

The present study goes beyond our previous findings inasmuch as it identifies venom HYAs as important targets of carbohydrate-specific IgE. Possibly, also HB venom acid phosphatase, which is known to be a glycoprotein [19] and which partly co-migrated with HYA in our gels, plays a role. Additionally, several other not yet identified IgE-binding glycoallergens could be located at 38 kDa in YJ venom, and in the HMW range in HB venom.

We assume that the HMW HB venom glycoallergens identified by us correspond to similar allergens described by previous researchers, irrespective of minor differences in reported molecular weights. Hoffman isolated by gel filtration four HMW allergens, different from HYA, with molecular weights of 49, 71, 87 and 105 kDa, the two most important of which he designated 'allergen B' (49 kDa, with acid phosphatase activity) and 'allergen C' (105 kDa) [31, 32]. A very similar HMW fraction was described by Karpas et al. [33]. Recently, Kettner et al. [34] re-investigated HMW HB allergens identifying proteins of 50, 69 and 94 kDa as important IgE-binding components in 63% of patients with HB venom allergy. N-terminal sequencing revealed distinct sequences for all proteins [34] excluding that they represent homopolymers of a single smaller protein [33].

We could not identify by mass spectrometry the glycosylated 38-kDa allergen detected in YJ venom; however, it is tempting to speculate that this allergen corresponds to V mac 3, a minor 39-kDa allergen originally described from *Vespa maculifrons* [11]. Likewise, the carbohydrate-specific antibodies binding around 100 kDa in YJ venom might identify homologues of V mac 1, another minor *V. maculifrons* allergen of unknown significance [11]. The proposed involvement of carbohydrate epitopes in antibody binding to the above-mentioned allergens fits well to Hoffman's observation that the sera from HB-*Vespa* double-positive patients specifically bound with HYA, V mac 1 and V mac 3 in *V. maculifrons* venom [11]. Furthermore, the reactivity with V mac 1 was inhibited by HMW HB venom allergens indicating a common antigenic determinant. The present study sheds new light on the nature and the potential clinical role of these poorly understood hymenoptera allergens. Carbohydrate may be the major and perhaps only target for IgE antibodies binding to these allergens. Accordingly, not all of these proteins might represent clinically important allergens to be considered in future immunotherapy [34]. However, further studies have to clarify whether or not these allergens bind with IgE only through their carbohydrates.

Surprisingly, HB PLA did not contribute much to the reactivity with carbohydrate-specific IgE, although binding of human IgE and rabbit IgG with its glycans has been demonstrated [22, 23, 35]. Comparatively weak reactivity of carbohydrate-specific antisera with ELISA plate-coupled

honeybee PLA has been described earlier [17]. PLA bears only one glycosylation site [36], but this is unlikely to be reason for the weak reactivity. Our data indicate that the allergen's tertiary structure could be a factor as we did see IgE binding after protein denaturation. In mouse major histocompatibility complex class II restricted T cell hybridomas against synthetic glycopeptides, single amino acid substitutions in the carrier peptide interfered with proper T cell receptor-mediated stimulation [37]. Hence, it can be hypothesized that, in a similar way, antibody binding to the PLA glycan is sterically hampered by adjacent amino acid residues in the differently folded native protein.

A certain limitation of our results derives from the difficulty to detect adequately all relevant YJ venom allergens in western blots. Non-reducing SDS-PAGE, which we used as the standard method, appeared to be superior to reducing SDS-PAGE with respect to the detection of YJ antigen 5 and PLA. The complete lack of IgE binding to antigen 5 in the reduced venom, and the weaker reactivity with PLA may be because of loss of conformational epitopes [38]. Likewise, the diminished reactivity with antigen 5 and PLA after periodate treatment observed in many patients may be caused by the destruction of conformational epitopes. On the other hand, in the non-reduced venom, HYA was found only in traces by mass spectrometry, and was poorly identified by patients' sera. The most plausible explanation for this is precipitation of HYA in the native venom essentially preventing its migration into the gel. Similar qualitative and quantitative differences between reducing and non-reducing electrophoresis have been described in the past by Hoffman [38], suggesting that not all IgE specificities can be accurately determined by either method.

In conclusion, we substantiated by immunoblot inhibition using two glycoprotein sources displaying fucosylated *N*-glycans the proposed role of carbohydrate-specific IgE as the cause of double positivity to HB and vespid venom in patients with stinging-insect allergy. The responsible allergenic glycans were located on venom HYAs and several other proteins some of which may correspond to previously described minor hymenoptera allergens with open clinical significance. Understanding that IgE against carbohydrates has low clinical relevance, our findings may improve the diagnosis of venom allergy because it should enable us to discriminate between relevant and irrelevant co-sensitization in double-positive patients. We may guess from this study that co-sensitization in pts 10 and 12–15, involving peptide epitopes, implicates the risk of an allergic reaction after a sting from either insect, whereas cross-sensitization through carbohydrates in the remaining patients (pts 1–9 and 11) can be regarded as not important.

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