Clin Exp Allergy 2005; **35**:441–447 doi:10.1111/j.1365-2222.2005.02217.x

Prevalence and clinical relevance of specific immunoglobulin E to pollen caused by sting- induced specific immunoglobulin E to cross-reacting carbohydrate determinants in Hymenoptera venoms

A.-M. Kochuyt, E. M. Van Hoeyveld and E. A. M. Stevens

Department of Internal Medicine, Division of Allergy and Clinical Immunology, University Hospital Gasthuisberg, Leuven, Belgium

Summary

Background Hymenoptera stings can induce specific IgE (sIgE) to carbohydrate determinants (CD) on venom glycoproteins that cross-react with CD in pollen. sIgE to such cross-reacting CD (CCD) are believed to have little or no biological activity and thus may cause misdiagnosis of pollen sensitization after a sting.

Objective To determine the prevalence of multiple false positive CAP results to pollen because of sting induced anti-CCD sIgE in Hymenoptera venom (HV) allergic patients and to investigate the association of such anti-CCD sIgE with features of 'atopy'.

Methods Skin prick tests (SPT) and CAP tests with grass, tree and weed pollen and with house dust mite (HDM) were carried out prospectively in 259 HV allergic patients and CAP tests with honeybee (HBV) and yellow jacket (YJV) venom were performed. Patients with negative pollen SPT associated with positive CAP tests to all three pollen groups were operationally defined as 'CCD positive'. We investigated in selected 'CCD positive' patients the presence of anti-CCD sIgE by CAP tests with bromelain and studied the identity of CD in HVs and pollen by mutual sIgE inhibition tests with CD from proteinase treated HBV (HBV-CD) and *Lolium perenne* (Lol-CD) extracts.

Results sIgE to all three pollen groups without positive SPT or history was found in 16% of 259 patients. The presence of anti-CCD sIgE was substantiated by positive CAP tests with bromelain in 14/14 and by inhibition of all pollen CAP tests with HBV-CD in 8/9 and with Lol-CD in 2/2 patients. Double venom (DV) positive CAP tests were present in 93% of 'CCD positive' patients and were in some associated with DV skin test positivity and allergy. The prevalence of 'CCD positivity' was significantly higher among HBV (23%) than among YJV (11%) allergic patients, but was also unexpectedly high among those with DV allergy (47%). 'CCD positive' patients were younger, had a higher total IgE and more sIgE to HDM than 'CCD negative' patients.

Conclusion We have shown that the risk in HV allergic patients for misdiagnosis of multivalent pollen sensitization is 16%, and we have confirmed that sting induced anti-pollen sIgE are directed to similar CD in venoms and pollen. We found evidence that the recognition of CCD might be related to the 'atopic' trait. Importantly, a positive bromelain CAP test does not exclude clinical reactivity to both venoms in 'CCD positive' HV allergic patients.

Keywords bromelain, cross-reacting carbohydrate determinants (CCD), false positive CAP tests, hymenoptera venom allergy, pollen allergy

Submitted 15 January 2004; revised 31 January 2005; accepted 02 February 2005

Introduction

The diagnosis of pollen allergy is based on history and on skin prick tests (SPT) which are, for practical reasons, often substituted by determination of specific IgE (sIgE) to pollen. In recent years, however, it has become clear that sIgE tests to pollen can be positive because of sIgE to cross-reacting carbohydrate determinants (CCD) [1–3]. Anti-CCD sIgE are not associated with positive SPT to pollen or with a positive clinical history and, therefore, lead to misdiagnosis of pollen allergy [3, 4]. Anti-CCD sIgE can be detected with the CAP test to bromelain, which contains on its carbohydrate moiety the β1,2-xylose and the α1,3-fucose residues that are present on many pollen glycoproteins [5–7]. A positive CAP test to bromelain is found in approximately one in three patients who demonstrate a positive SPT to one or more pollen species associated with discrepant SPT–CAP results to the other pollen species [8]. Carbohydrate structures carrying the α1,3-fucose residue are also present on Hymenoptera venom (HV) glycoproteins [9, 10] and, consequently, anti-CCD sIgE may appear after a sting. An association between sIgE [1] or sIgG

Correspondence: A.-M. Kochuyt, Department of Internal Medicine, Division of Allergy and Clinical Immunology, University Hospital Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium.
E-mail: Anne-Marie.Kochuyt@uz.kuleuven.ac.be

© 2005 Blackwell Publishing Ltd

[11] to pollen and to honeybee venom (HBV) has been described as early as in 1981, whereas the association between sIgE to pollen and to bumblebee venom (BBV) has been reported by our group in 1993 [12]. Such association has originally been attributed to a dual sensitization to pollen and to HV and, therefore, related to a proneness for multiple sensitizations as is seen in allergic individuals [13]. However, alternatively it may be caused by induction of anti-CCD sIgE by insect stings as was already proposed by Aalberse et al. [1]. This has not been investigated, so far, in an HV allergic population. We intended in this study to examine the extend of the risk of misdiagnosis of pollen allergy because of sting induced anti-CCD sIgE. We, therefore, carried out a prospective clinical study in 259 HV allergic patients using history, SPT and CAP tests with grass, tree and weed pollen. The presence of anti-CCD IgE was demonstrated with bromelain CAP tests and with CAP inhibition tests using carbohydrate preparations from pollen and HBV extracts. We in addition explored whether anti-CCD sIgE were associated with markers of atopy.

Materials and methods

Patients

The study was carried out in 259 consecutive subjects referred to our Division of Allergy and Clinical Immunology because of a systemic reaction (SR) to an insect sting. Fifty-two patients (24 females, 28 males) had reacted to a honeybee (HB) sting and 190 patients (86 females, 104 males) to a yellow jacket (YJ) sting. Seventeen patients (five females, 12 males) had reacted with a SR to a sting of one of both insects and a large local reaction (LLR) >10 cm to the other ($n = 13$) or with a SR to both HB and YJ stings ($n = 4$). The median age of the 259 patients was 41 yrs (range: 6–70 years). There was no age difference between the patients with allergic reactions to HBV, to yellow jacket venom (YJV) or to both venoms. All patients were proven to be sensitized to a HV by a positive intracutaneous skin test (ICST) and a positive sIgE determination (CAP) for YJV and/or HBV. CAP tests with HBV and YJV and both SPT and CAP tests with pollen and house dust mite (HDM) allergens were performed prospectively. Patients were questioned for the presence of rhinitis and asthma. Patients with negative SPT to pollen were defined as 'CCD positive' when they had positive CAP tests to all three pollen groups and as 'CCD negative' when CAP tests to all three pollen groups were negative. Blood samples were stored at $-20\,^{\circ}$C.

Skin tests

ICST with the culprit venom were performed in all patients and were carried out with 0.05 mL of 10^{-6} to 10^{-1} µg/mL solutions of a commercial YJV or HBV extract (Pharmalgen®, Pharmacia, Sweden). ICST with both venoms were only performed in case of doubt on the identity of the insect that had caused a SR or on request of the patient. With respiratory allergens, SPT were carried out with a screening panel including pollen of grasses (*Agrostis stolonifera*, *Anthoxanthum odoratum*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*), weeds (*Artemisia vulgaris*, *Chenopodium album*, *Plantago lanceolata*) and trees (*Alnus incana*, *Betula verrucosa*, *Corylus avellana*, *Fagus grandifolia*) and HDM in all patients. Skin test extracts were from Hal, The Netherlands. The skin test reactions were read after 15 min and graded according to the method of Voorhorst et al. [14].

Laboratory investigations

Total IgE and sIgE Total IgE and sIgE were determined using the CAP system (Pharmacia-Upjohn, Sweden). Tests were performed according to the manufacturer's instructions. For sIgE a CAP value of >0.34 Ua/mL was considered positive. Specific IgE to HBV, YJV and to a panel of respiratory allergens was measured in all patients. The panel consisted of a mixture of common grass pollen (g × 3: *A. odoratum*, *Holcus lanatus*, *L. perenne*, *P. pratense*, *Secale cereale*), tree pollen (t × 6: *Acer negundo*, *B. verrucosa*, *Fagus grandifolia*, *Juglans californica*, *Quercus alba*), weed pollen (w × 5: *Ambrosia elatior*, *A. vulgaris*, *Chrysanthemum leucanthemum*, *Solidago vigaurea*, *Taraxacum vulgare*) and HDM. A CAP test with the glycoprotein bromelain, which was not commercially available at the start of the study, was performed consecutively in 14 'CCD positive' and 23 'CCD negative' patients.

Honeybee venom and Lolium perenne *extracts* Electrically collected pure whole HBV 100 µg/mL (*Apis mellifera*) was purchased from Allergon (Ängelholm, Sweden) (Vespa Laboratories, Spring Mills, PA, USA). *L. perenne* pollen were purchased from Allergon. An extract of *L. perenne* pollen was prepared 1/10 (w/v) in distilled water for 3 h at room temperature with continuous shaking and a constant pH adjustment as described by Cadot et al. [15].

Source of CD Proteinase K-treated HBV and proteinase K-treated grass pollen (*L. perenne*) were used as a source of CD for CAP and immunoblot inhibition experiments. Extracts were incubated with proteinase K (*Titrirachium album*; Boehringer Mannheim, Mannheim, FRG)(1 mg/22.4 µg) for 8 h at 50 °C. Proteinase K was subsequently inactivated for 5 min at 100 °C. Intact protein material in the digested HBV and *L. perenne* extracts was undetectable in the Coomassie blue assay (Bio-Rad, Richmond, CA, USA) carried out according to the manufacturer's instructions, and by SDS-PAGE.

SDS-PAGE was carried out in 12% polyacrylamide gels with a 5% polyacrylamide stacking gel, according to Laemmli. Samples were heated for 5 min at 100 °C in sample buffer (125 mmol/L Tris pH 6.8%, 17.5% glycerol, 4% SDS, 1% β-mercaptoethanol and 0.002% bromophenol blue). Gels were stained with Coomassie blue.

The determination of the optimal amount of proteinase K digested extracts for the CAP inhibition studies were limited by the viscosity of the material at higher concentrations. CD obtained from 1 mg HBV (HBV-CD) and from 0.1 mg *L. perenne* extract (Lol-CD) were used in all experiments.

Inhibition of CAP tests to pollen and venoms with HBV-CD, Lol-CD, whole HBV Serum (25 µL) was preincubated with 25 µL of HBV-CD derived from 1 mg/mL HBV, with

25 μL of Lol-CD derived from 0.1 mg/mL *L. perenne* extract, with 25 μL of whole HBV (100 μg/mL) or with 1% HSA. After 2 h at room temperature, serum was added to CAPs coupled with a grass pollen, tree pollen or weed pollen mixture or with YJV or HBV and incubated for 30 min. The CAPs were then washed and incubated for 2 h 30 min with 50 μL radio-iodinated anti-IgE antibodies (Pharmacia-Upjohn, Sweden). They were washed before reading in a γ-counter. The inhibition of IgE binding of the serum preincubated with proteinase K treated extract was calculated as a percentage of the IgE binding of the serum preincubated with HSA. Tests were carried out in duplicate. A preliminary experiment demonstrated that the minimal serum sIgE level necessary to obtain reproducible results in our inhibition studies was 4 Ua/mL. Therefore all sera used had sIgE values >4 Ua/mL.

Statistical methods

Statistical analyses were performed either by the χ^2 test, the Fischer's test, the Mann–Whitney *U*-test, Spearman's rank correlation or *T*-test.

Results

Prevalence of positive SPT or CAP tests with pollen or HDM in 259 patients with HV anaphylaxis

SPT or CAP tests were positive with pollen or HDM in 28% and 44% of the 259 patients, respectively (Table 1), whereas manifest allergic rhinitis or asthma were mentioned by only 17% (data not shown). SPT with grass, tree or weed pollen were positive in 14% but CAP tests with any of these pollen in 34% of the patients, resulting in a discrepancy between negative SPT associated with one or more positive CAP tests with pollen in as many as 68 of the 259 patients (26%). In these 68 patients, discrepant SPT–CAP results were found in 27 subjects for only one or two pollen groups and in 41 subjects for all three pollen groups with very similar CAP values for the three pollen groups in the individual patient. Because in HV allergic patients, a SPT–CAP discrepancy involving all three pollen mixtures is suggestive for the presence of sting-induced anti-CCD sIgE these 41 patients were operationally defined as 'CCD positive'. This resulted in a prevalence of 16% 'CCD positivity' which was only slightly reduced to 13% when changing the cut-off for CAP test positivity from 0.35 to 0.70 Ua/mL for all three pollen mixtures. The clinical irrelevance of anti-CCD IgE was substantiated by a negative nasal provocation test with grass pollen in 6/6 ($P<0.04$) such 'CCD positive' patients with high sIgE levels to pollen, and, in addition, by the observation that no symptoms typical for pollen allergy were ever mentioned by any of our 'CCD positive' patients during a follow-up of several years.

Table 1. Prevalence of positive SPT or positive CAP tests to pollen or HDM, one to two pollen groups or all three pollen groups in 259 Hymenoptera venom allergic patients

	SPT positive *n* (%)	CAP test positive *n* (%)	SPT negative/CAP test positive *n* (%)
Pollen or HDM	72 (28)	113 (44)	77 (30)
Grass or tree or weed pollen	36 (14)	87 (34)	68 (26)
Grass and tree and weed pollen	5 (2)	52 (20)	41* (16)

*Operationally defined as 'CCD positive' patients.
SPT, skin prick test; HDM, house dust mite; CCD, cross-reacting carbohydrate determinants.

In the group of 27 patients with SPT–CAP discrepancies for only one or two pollen groups, sIgE to CD in pollen or venoms may have been present in 20 patients. Nine subjects had positive SPT with at least one pollen group associated with SPT–CAP discrepancies for the other pollen. In these nine patients the IgE to CD may have been elicited by pollen rather than by venom, and therefore they were not included in the sting-induced 'CCD positive' group. Another 11 patients, who were SPT negative for the tested pollen, had a CAP pattern that was compatible with anti-CCD IgE, because they all demonstrated sIgE to grass pollen which were in 4 of them associated with slightly lower sIgE levels to tree pollen. If we would still incorporate these 11 subjects in the 'CCD positive' group the prevalence of sting-induced anti-CCD IgE would increase only from 16% to 20%.

The 163 patients with negative SPT and negative CAP tests to all these pollen were defined as 'CCD negative'.

Evidence for sting-induced anti-CCD sIgE in 'CCD positive' patients

CAP tests with the glycoprotein bromelain CAP tests with bromelain were carried out consecutively in 14 'CCD positive' and 23 'CCD negative' patients. No patient had an allergy to pineapple. CAP tests were positive in all 14 'CCD positive' patients (range: 0.49–10.8 Ua/mL, median: 2.31 Ua/mL) (Table 2) and only borderline positive in one 'CCD negative' patient (0.36 Ua/mL). In the majority of the patients the sIgE level to bromelain was of the same order of magnitude as the sIgE levels to the different pollen groups and to the non-culprit venom (Table 2).

Inhibition of pollen CAP tests with HBV-CD, Lol-CD or whole HBV HBV-CD were used for inhibition of pollen CAP tests in seven 'CCD positive' patients (Table 3). In six patients pollen CAP tests were inhibited by 19–100% (median 66% for 18 inhibition tests). In one patient (no 6) sIgE to pollen could not be inhibited. Lol-CD were used for inhibition of pollen CAP tests in two 'CCD positive' patients (Table 3). The percentage of inhibition ranged from 29% to 87% (median 60.5% for six inhibition tests). These results demonstrate that CD from HBV and from pollen are able to inhibit to the same extend the IgE binding to pollen in 'CCD positive' patients. Whole HBV was used as reference inhibitor in two 'CCD positive' patients and was able to inhibit pollen CAP tests by 85–97% (median 90% for six inhibition tests).

Inhibition of pollen CAP tests with HBV-CD were also carried out in three control subjects with a clinically relevant, SPT positive, allergy to grass pollen associated with negative CAP tests to tree and weed pollen. The HBV-CD extract did not inhibit the grass pollen CAP tests (data not shown)

Table 2. CAP tests to bromelain, insect venoms and pollen in 14 'CCD positive' Hymenoptera venom allergic patients

Patient	Culprit Venom	CAP test (Ua/mL) to						Total IgE (KU/L)
		Bromelain	HBV	YJV	GP	TP	WP	
DP	YJ	0.49	0.50	1.96	1.93	0.50	0.53	521
VLG	YJ+HB	0.52	11.70	0.62	0.49	0.40	0.40	26
JA	YJ	0.66	0.68	6.26	0.56	0.53	0.51	208
LH	HB	1.40	>100	0.93	1.93	1.43	1.24	350
CF	YJ	1.42	2.26	11.50	1.35	0.85	0.60	335
LS	HB	2.05	27.70	2.52	3.88	3.32	2.35	161
EH	HB	2.23	20.90	0.79	2.48	1.85	1.74	140
RG	HB	2.39	5.76	0.53	1.96	1.06	0.77	50
BH	YJ+HB	5.77	30.2	9.92	5.49	4.59	4.45	103
GL	YJ+HB	7.45	98	5.08	7.46	6.25	6.10	—
BR	YJ	8.28	2.45	29.60	2.61	0.77	0.50	879
SM	HB	9.70	>100	2.21	8.14	3.90	4.44	753
DP	YJ+HB	10.80	37	11.30	15.80	12.50	10.40	1273
VJ	YJ	10.80	12	32	11.10	9.03	8.82	420

HBV, honeybee venom; GP, grass pollen mixture; —, not done; YJV, yellow jacket venom; TP, tree pollen mixture; WP, weed pollen mixture; CCD, cross-reacting carbohydrate determinants.

Table 3. Inhibition of grass, tree and weed pollen CAP tests with HBV-CD, Lol-CD or whole HBV in nine 'CCD positive' Hymenoptera venom allergic patients

Patient No	CAP tests (Ua/mL) to pollen of			Inhibition (%) of CAP tests to pollen of		
	Grasses	Trees	Weeds	Grasses	Trees	Weeds
				with HBV-CD		
1	>100	>100	82.7	65	66	76
2	10.6	12.2	20.2	60	69	87
3	6.9	6.3	5.2	19	66	32
4	6.7	7.1	5.7	86	100	94
5	6.5	6.5	5.6	34	47	57
6	6.9	6.8	5.8	0	0	0
7	7	5.7	4.7	80	93	96
				with Lol-CD		
8	50	43	44	29	60	38
9	14.5	13.7	11	61	87	78
				with whole HBV		
8	50	43	44	85	92	87
9	14.5	13.7	11	87	97	92

HBV, honeybee venom; CD, carbohydrate determinants; CCD, cross-reacting carbohydrate determinants; Lol, *Lolium perenne*.

showing that patients with a SPT and CAP test monovalent pollen allergy do not recognize CCD.

Serial determinations of the sIgE levels to pollen and venoms (data not shown) The sting origin of anti-CCD sIgE in 'CCD positive' patients was substantiated by serial sIgE determinations in three patients showing that negative or very low anti-CCD IgE levels before a sting increased in parallel with the culprit venom sIgE after the sting. In three other patients, clear positive anti-CCD IgE levels after a sting had decreased considerably several months later in parallel with the culprit venom sIgE. Seasonal pollen exposure was on the other hand not associated with boosting of the anti-pollen sIgE levels in 'CCD positive' patients.

Comparison of 'CCD positive' and 'CCD negative' HV allergic patients for features related to atopy

Compared to the 163 'CCD negative' patients the 41 'CCD positive' patients were significantly younger (median age: 45 and 36 years, respectively, $P<0.03$) and had a higher total IgE value (exp(ln(IgE)): 75 and 270 kU/L, respectively, $P<0.001$). The prevalence of positive SPT with HDM (15% and 14%) was not different but a positive CAP test for HDM associated with a negative SPT was found more often in the 'CCD positive' (17%) than in the 'CCD negative' group (4%) ($P<0.02$), even when increasing the cut-off for a positive HDM CAP test from 0.35 to 0.70 Ua/mL. The sIgE levels to HDM were not related to the total IgE level that makes false positive results because of non-specific IgE binding improbable.

Comparison of 'CCD positive' and 'CCD negative' HV allergic patients for DV positive CAP tests

DV positive CAP tests were found in 38/41 (93%) 'CCD positive' patients and in only 22/193 (13.5%) 'CCD negative' patients ($P<0.001$). Of these 38 patients, eight mentioned allergic reactions to both venoms and 30 to a single venom. However, only half of these 30 subjects had a recollection of having been stung by both insects. To demonstrate that in patients with a clinical single venom allergy anti-CCD sIgE contributed to the positivity of the CAP tests to non-culprit venoms, we tried to inhibit these venom CAP tests with HBV-CD and Lol-CD. Sera from six 'CCD positive' patients with YJV ($n=4$) allergy (non-culprit venom: HBV) or BBV allergy (non-culprit venom: HBV and YJV) were selected. The percentage of inhibition ranged from 44% to 94% (median 80% for nine inhibition tests) (Table 4a). Anti-CCD sIgE antibodies appearing after a sting are elicited by CCD present on glycoproteins of the culprit venom. We therefore would expect at least a partial inhibition of the culprit venom CAP by CD in 'CCD positive' patients. However YJV CAP tests could not be inhibited with HBV-CD in two out of three

Table 4. Inhibition of venom CAP tests with HBV-CD, Lol-CD, or whole HBV in 'CCD positive' Hymenoptera venom allergic patients

Patient No	Culprit insect	Reaction grade†	Cap* non-culprit venom	Inhibition (%) of CAP
(a) Inhibition of non-culprit venom CAP tests in six single venom allergic patients				
				with HBV-CD
1	YJ	IV	>100 (HB)	80
2	YJ	IV	24 (HB)	94
4	BB	II	30 (HB)	85
			7.5 (YJ)	73
5	BB	I	9.2 (HB)	67
			4.0 (YJ)	92
8	YJ	III	48 (HB)	59
				with Lol-CD
8	YJ	III	48 (HB)	44
9	YJ	III	12 (HB)	85
				with whole HBV
8	YJ	III	48 (HB)	92
9	YJ	III	12 (HB)	97
(b) Inhibition of culprit venom CAP tests in three double venom allergic patients				
				with HBV-CD
3	HB	III	6.8	37
	YJ	IV	5.4	0
6	HB	III	>100	0
	YJ	LLR	25	0
7	HB	III	>100	8
	YJ	II	11.9	36

*Ua/mL.
†According to Mueller.
HB, honeybee; YJ, yellow jacket; BB, bumblebee; HBV, honeybee venom; CD, carbohydrate determinants; CCD, cross-reacting carbohydrate determinants; Lol, *Lolium perenne*.

'CCD positive' YJV allergic patients (data not shown). This demonstrates that as far as the culprit venom is concerned the sIgE binding to peptides is highly dominant over the sIgE binding to CCD, which thus represents only a small fraction of the IgE response to the culprit venom.

Comparison of 'CCD positive' and 'CCD negative' HV allergic patients for DV positive ICST

ICST with both venoms were performed in 17 CCD positive-DV CAP positive patients and in 19 CCD negative -single venom CAP positive patients and were positive with both venoms in 13 (76%) and six (32%) ($P<0.02$) patients, respectively, when including 1 μg/mL, and in six (35%) and 0 patients, respectively, when testing up to 0.1 μg/mL ($P<0.02$). About half of the CCD positive patients with DV positive ICST had a DV positive history.

Prevalence of 'CCD positivity' in patients with clinical allergy to HBV, YJV or both venoms

As shown in Table 5 the frequency of 'CCD positivity' was significantly higher in HBV allergic (23%) than in YJV allergic patients (11%) and was also higher in the 17 patients with DV clinical allergy, of whom eight (47%) were 'CCD positive', than in the YJV allergic patients. In these eight patients the pollen CAP tests were positive but clinically irrelevant, whereas the DV positive CAP tests were apparently both clinically relevant. Indeed, two patients had experienced SRs to both HBV and YJV, whereas the remaining six patients mentioned a SR to one venom and one or more LLR to the other venom. We, therefore, investigated the contribution of anti-CCD sIgE to the positivity of the venom CAP tests in three such patients by inhibiting CAP tests to both HBV and YJV with HBV-CD. As shown in Table 4b, no or a very modest inhibition was obtained, suggesting that the sIgE binding to both venoms was predominantly directed to peptides. The finding that both venoms behaved like culprit venoms in this inhibition experiment is in agreement with the clinical history of these 'CCD positive' patients.

Table 5. Prevalence of 'CCD positivity' in patients with clinical allergy to honeybee (HBV), yellow jacket (YJV) or both venoms

	"CCD positive"				
Allergic to	***N***	***n***	***(%)***	***p***	
HBV	52	12	(23)	$<0.05^{\dagger}$	$<0.12^{\ddagger}$
YJV	190	21	(11)		
HBV + YJV	17	8	(47)	$<0.001^{\ddagger}$	
Total	259	41	(16)		

†χ^2 test.
‡Fischer' test.
HBV, honeybee venom; YJV, yellow jacket venom; CD, carbohydrate determinants; CCD, cross-reacting carbohydrate determinants.

Discussion

In the first part of this study we investigated the prevalence of anti-CCD sIgE induced by a sting and causing false positive *in vitro* diagnosis of multivalent pollen allergy. Negative SPT with positive CAP tests for all three pollen groups were used as the marker for sting induced anti-CCD sIgE and were found in 41 (16%) of 259 HV allergic patients (Table 1). Theoretically it might be possible that anti-CCD IgE were pre-existent to the sting and that these 41 'CCD positive' patients were primarily sensitized to pollen glycans independently from pollen peptides. However, this is very unlikely for different reasons. At first, preliminary experiments (data not shown) demonstrated that the sIgE to the three pollen mixtures increased after a sting and evolved in parallel with the sIgE to both culprit and non-culprit venoms which strongly argues for the sting origin of the anti-CCD IgE. In addition, we observed no boosting of the sIgE levels to the three pollen groups during or after the pollen season, which is an argument against a sensitization to pollen glycans via the respiratory route. SPT–CAP discrepancies for only one or two pollen groups were present in another 27 patients but were in only 11 of them suggestive of sting induced anti-CCD IgE. Indeed, in the remaining 16 patients SPT–CAP discrepancies could have been caused by pollen induced anti-CD IgE in nine patients with positive pollen SPT or by

IgE to a low skin reactive pollen peptide in the 11 remaining subjects. The prevalence of 16% 'CCD positivity', mimicking multivalent pollen sensitization, may thus well underestimate the prevalence of sting induced anti-CCD sIgE, which may be as high as 20%, and consequently the risk for false positive diagnosis of any pollen allergy in HV allergic patients. Importantly, a true pollen allergy with positive SPT, was found in only 14% of our study patients (Table 1) and was accompanied by symptoms in 75% of them. Our results, therefore, strongly suggest that the association between sIgE to pollen and HVs, which was reported in former clinical studies [11, 13], is for a major part because of anti-CCD sIgE, as was also suggested by Hemmer et al. [16] and not to a proneness of pollen allergics to become sensitized to HV peptides or vice versa. This is an important notion because otherwise pollen allergics should be dissuaded from occupational or other contacts with insects. We, in addition, did not observe a higher prevalence of respiratory allergy, defined as a positive SPT to HDM or pollen (Table 1) associated with rhinitis or asthma, than in a general population [17, 18, 19]. This confirms the results of an earlier study that respiratory allergy does not predispose for HV allergy [20].

In the second part of our study we demonstrated that the anti-pollen sIgE in the serum of our 'CCD positive' patients are indeed directed to CCD and we investigated to what extend anti-CCD sIgE are responsible for the pollen CAP test positivity. The bromelain CAP test, that is currently used for the detection of anti-CCD sIgE detects sIgE to the α1,3-fucose residue, present in venoms and pollen and to the β1,2-xylose residue, uniquely present in pollen [5, 6, 7, 9]. All 14 tested 'CCD positive' patients had sIgE to bromelain (Table 2) and because all true pollen allergics had been excluded, the sIgE to bromelain could only be directed to CD in venom cross-reacting with CD in pollen. In addition, we found a moderate to strong inhibition of the sIgE binding to all pollen groups by pre-incubation of serum with HBV-CD in 6/7 and with Lol-CD in 2/2 'CCD positive' patients (Table 3), but no inhibition of the sIgE binding to grass pollen in true pollen allergics. The extend of the inhibitions was very variable as was also noticed by others [3, 16]. As an explanation for the lower inhibition percentages in our patients 3, 5 and 6, we might suggest competition of anti-CCD sIgE with anti-peptide sIgE that is not biologically active or an insufficient quantity of inhibitory CD in the CD preparation used (as discussed in materials and methods). However, the observation that much higher inhibition percentages were obtained with whole HBV indicates that intact glycan-peptide epitopes which were damaged by the proteinase treatment of the HBV extract are needed for IgE-binding. In the majority of 'CCD positive' patients the CAP test values as well as the degree of inhibition with CD were very similar for the three pollen groups in the same patient (Tables 2 and 3). This suggests that the pollen mixtures on the CAP tests contain similar amounts of CCD.

Anti-CCD sIgE occur in up to 31% of pollen allergic patients with at least one positive pollen SPT [8]. In patients with such mixed IgE response to peptides and CD in pollen it is difficult to prove the clinical irrelevance of anti-CCD IgE. The situation is obviously different for HV allergic patients with sting-induced anti-pollen sIgE that is almost exclusively directed to CD. None of our 'CCD positive' patients actually had symptoms of pollen allergy. The absence of clinical reactivity to pollen in such patients was proven by nasal provocation tests in 6/6 subjects ($P<0.04$) and substantiated by a follow-up of several years which showed that no 'CCD positive' patient developed hay fever. We thus demonstrated that anti-CCD sIgE are not associated with pollen allergy. Patients with anti-CCD sIgE are not only at risk for false positive diagnosis of pollen allergy but also for misdiagnosis of venom allergy. Hemmer et al. recently reported that anti-CCD sIgE is a frequent cause of false positive CAP tests with venoms and emphasized the risk for erroneous interpretation of these CAP tests when the culprit insect has not been identified [16]. In our study as many as 38 of our 41 'CCD positive' patients had sIgE to both HBV and YJV. The majority of them did not remember having been stung by both insects, which makes it impossible to assess the clinical relevance of those DV positive CAP tests in the whole group. The sIgE levels to pollen and to both venoms were significantly correlated (rs: 0.43, $P<0.001$ for the culprit venom and rs: 0.43, $P<0.02$ for the non-culprit venom) which again argues against a separate sensitization to pollen glycans. Furthermore, the sIgE levels to pollen and to the non-culprit venom were remarkably similar but significantly lower than those to the culprit venom ($P<0.01$) suggesting that the sIgE were predominantly directed to CCD in non-culprit venoms and predominantly to peptides in culprit venoms. These data are in agreement with results of our inhibition experiments: CAP tests to the non-culprit venom were substantially inhibited with both HBV-CD and Lol-CD (Table 4a) whereas CAP tests to the culprit venom were almost not (data not shown). Interestingly, Hemmer et al. [16] did not either obtain substantial inhibitions of culprit venom CAP tests with MUXF, a neoglycoprotein containing the bromelain glycan, in 6/10 of his patients. Although in his study [16], none of the patients with DV positive CAP tests had a DV positive clinical reactivity, six of our 17 'CCD positive' patients who were skin tested with both venoms, had clear DV positive ICST and half of them had experienced allergic reactions to both venoms. Likewise eight of the 17 patients with a DV positive history had anti-CCD sIgE (Table 5). Three such patients were further investigated and the clinical relevance of the DV positive CAP tests was proven by the fact that neither venom CAP could be substantially inhibited with HBV-CD (Table 4b). Similar low inhibitions ($<30\%$) of the IgE binding to both HBV and YJV were observed with MUXF in 4/10 of Hemmer's patients [16]. This illustrates that some 'CCD positive' patients may have sIgE to both CCD and peptides in different venoms as was also recently demonstrated on immunoblot [10]. For the clinical practice these observations signify that in bromelain positive patients DV positive CAP tests should not automatically be interpreted as a clinical irrelevant consequence of anti-CCD sIgE.

We tried to determine factors predisposing for the presence of anti-CCD sIgE. The sting frequency did not seem to be of influence because there was no relation between CCD positivity and the number of previous HB or YJ stings (data not shown). It is possible that the degree of glycosylation of venom allergens, which in particular for HBV is known to be high [10, 21], plays a role in the induction of anti-CCD sIgE. Although there are, when expressed in absolute number, more

'CCD positive' YJV than HBV allergics among our 259 patients, the prevalence of CCD positivity is significantly higher in patients with HBV (23%) allergy than in those with YJV allergy (11%) (Table 5) and the frequency is even higher in a group of 42 BBV allergics (43%) (data not shown). A similar high frequency (28%) of anti-glycan IgE was found in HBV allergics by Tretter et al. [9]. This indicates that HBV which partially cross-reacts with BBV, contains a higher amount of CD than YJV allergens as was also suggested by Hemmer's inhibition experiments [16]. Compared with 'CCD negative' patients, 'CCD positive' patients were younger ($P<0.03$) and had higher total IgE levels ($P<0.001$) that were not correlated with the sIgE levels to pollen which thus excludes non-specific IgE binding to the pollen CAPs. Mari [8] reported similar differences in age and IgE production for pollen allergic patients with and without anti-CCD sIgE and in addition associated anti-CCD IgE with the risk of developing multiple allergies. We found in 'CCD positive' patients a higher prevalence of sensitization to HDM by CAP but not by SPT, than in the 'CCD negative' patients. The reason for this CAP-SPT discrepancy for HDM, which was also observed by Mari [8], is not clear at present because a cross-reactivity between glycans of HDM and HV has not been described. Taken together, our results suggest that atopy, a condition that is associated with a tendency to produce sIgE to many different allergens, might be a facilitating factor for anti-CCD sIgE production after a sting.

In conclusion, we have shown that 23% and 11% of HBV and YJV allergic patients, respectively, have CAP tests to all pollen that are clinically false positive because of sting induced sIgE to CCD in HVs. It is interesting to speculate on the importance of the risk for misdiagnosis by sting-induced anti-CCD sIgE at the community level. Because 15–25% of all people ever stung – with or without reaction – produce anti-venom sIgE [13, 22, 23, 24] and at least 16% of this subgroup produces anti-CCD IgE antibodies, we can easily calculate that 2.4–4% of all subjects stung are at risk for misdiagnosis of allergy to pollen and non-culprit venoms. In countries where many people are stung, in particular by HBs, the occurrence of false positive pollen CAP tests in the general population will be high and this may seriously affect the outcome of epidemiological studies that are uniquely based on the results of sIgE determinations to pollen [25, 26].

References

1 Aalberse RC, Koshte B, Clemens JGJ. Immunoglobulin E antibodies that cross react with vegetable foods, pollen, and Hymenoptera venom. J Allergy Clin Immunol 1981; 68:356–64.

2 Iacovacci P, Pini C, Afferni C et al. A monoclonal antibody specific for a carbohydrate epitope recognizes an IgE-binding determinant shared by taxonomically unrelated allergenic pollens. Clin Exp Allergy 2001; 31:458–65.

3 Mari A, Iacovacci P, Afferni C. Specific IgE to cross reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. J Allergy Clin Immunol 1999; 103:1005–11.

4 Van der Veen MJ, van Ree R, Aalberse RC. Poor biologic activity of cross reactive IgE directed to carbohydrate determinants of glycoproteins. J Allergy Clin Immunol 1997; 100:327–34.

5 Afferni C, Iacovacci P, Barletta B et al. Role of carbohydrate moieties in IgE binding to allergenic components of Cupressus arizonica pollen extract. Clin Exp Allergy 1999; 29:1087–94.

6 Van Ree R, Cabanes-Macheteau M, Akkerdaas J et al. Beta (1,2)-xylose and alpha (1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. J Biol Chem 2000; 275: 11451–8.

7 Wilson IB, Altmann F. Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens: core alpha1,3-linked fucose and xylose present in all pollens examined. Glycoconj J 1998; 15:1055–70.

8 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; 129:286–95.

9 Tretter V, Altmann F, Kubelka V, März L, Becker WM. Fucose α1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honey bee venom allergic individuals. Int Arch Allergy Immunol 1993; 102:259–66.

10 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; 34:460–9.

11 Kemeny DM, Miyachi S, Platts-Mills TAE, Wilkins S, Lessof MH. The immune response to bee venom. Comparison of the antibody response to phospholipase A2 with the response to inhalant allergens. Int Arch Allergy Appl Immunol 1982; 68:268–74.

12 Kochuyt AM, Van Hoeyveld E, Stevens EAM. Occupational allergy to bumblebee venom. Clin Exp Allergy 1993; 23:190–5.

13 Schäfer T, Przybilla B. IgE antibodies to Hymenoptera venoms in the serum are common in the general population and are related to indications of atopy. Allergy 1996; 51:372–7.

14 Voorhorst R, Spieksma FTH, Varekamp H. House dust atopy and the house dust mite. Leiden: Stafleu, 1969.

15 Cadot P, Lejoly M, Van Hoeyveld EM, Stevens EAM. Influence of the pH of the extraction medium on the composition of birch (*Betula verrucosa*) pollen extracts. Allergy 1995; 50:431–7.

16 Hemmer W, Focke M, Kolarich D et al. Antibody binding to venom carbohydrates is a frequent cause for double venom positivity to honeybee and yellow jacket venom in patients with stinging-insect allergy. J Allergy Clin Immunol 2001; 108:1045–52.

17 Baldacci S, Modena P, Carozzi L et al. Skin prick test reactivity to common aeroallergens in relation to total IgE, respiratory symptoms, and smoking in a general population sample of Northern Italy. Allergy 1996; 51:149–56.

18 Plaschke P, Janson C, Norrman E et al. Skin prick tests and specific IgE in adults from three different areas of Sweden. Allergy 1996; 51:461–72.

19 Norrman E, Rosenhall L, Nyström L, Jönsson E, Stjernberg N. Prevalence of positive skin prick tests, allergic asthma, and rinoconjunctivitis in teenagers in northern Sweden. Allergy 1994; 49:808–15.

20 Birnbaum J, Vervloet D, Charpin D. Atopy and systemic reactions to Hymenoptera stings. Allergy Proc 1994; 15:49–52.

21 Kettner A, Henry H, Hughes GJ, Corradin G, Spertini F. IgE and T-cell responses to high-molecular weight allergens from bee venom. Clin Exp Allergy 1999; 29:394–401.

22 Golden DBK, Marsh DG, Kagey-Sobotka A et al. Epidemiology of insect venom sensitivity. JAMA 1989; 262:240–4.

23 Charpin D, Birnbaum J, Vervloet D. Epidemiology of hymenoptera allergy. Clin Exp Allergy 1994; 24:1010–5.

24 Jarisch R, Zajc J, Buzath A. The risk of sensitization of nonallergic persons to bee venom. Arch Dermatol Res 1982; 273:173–4.

25 Ezeamuzie CI, al-Mousawie M, Dashti H, al-Bashir A, al-Hage M, al-Ali S. Prevalence of allergic sensitization to inhalant allergens among blood donors in Kuwait, a desert country. Allergy 1997; 52:1194–200.

26 Sabina I, von Mutius E, Lau S et al. The pattern of atopic sensitization is associated with the development of asthma in childhood. J Allergy Clin Immunol 2001; 108:709–14.