

Reassessing the role of hyaluronidase in yellow jacket venom allergy

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Background: Yellow jacket hyaluronidase (YJ-HYA) is considered a major allergen in yellow jacket allergy. It shows 50% homology with the hyaluronidase from honeybee venom, Api m 2. Recently, IgE binding to YJ-HYA and cross-reactivity with Api m 2 has been shown to be due to cross-reactive carbohydrate determinants (CCDs).

Objective: We sought to quantify the importance of YJ-HYA in yellow jacket allergy and the cross-reactivity with Api m 2 by discriminating between carbohydrate and peptide epitopes.

Methods: IgE binding to *Vespula* species venom was studied by means of Western blotting in 136 patients with yellow jacket allergy (31 *in vitro* single positive to yellow jacket venom and 105 *in vitro* double-positive to yellow jacket-honeybee).

Inhibition studies were carried out with MUXF-BSA (isolated bromelain glycopeptides linked to bovine serum albumin) and purified Api m 2.

Results: Among yellow jacket single-positive sera, only 1 of 31 bound with YJ-HYA, whereas this was the case in 87% of 105 double-positive sera. Of 83 patients in whom inhibitions were performed, 65% reacted with hyaluronidase through CCDs alone, 27% reacted with both CCDs and peptide epitopes, and 8% reacted only with the hyaluronidase peptide. The protein-specific reactivity with YJ-HYA was cross-inhibited by Api m 2 in 48% (14/29). Antigen 5 and phospholipase A₁ were each recognized by around 90% of sera from both groups, together identifying 97% of patients.

Conclusion: Hyaluronidase is a minor yellow jacket venom allergen, and only 10% to 15% of patients with yellow jacket allergy are estimated to have IgE against the hyaluronidase protein. Peptide-specific cross-reactivity with Api m 2 occurs in half of these sera. Component-resolved diagnosis with antigen 5 and phospholipase would detect virtually all patients with yellow jacket venom allergy. (J Allergy Clin Immunol 2009;123:1016-1021.)

Key words: α 1,3-Fucose, cross-reactive carbohydrate determinants, honeybee, hyaluronidase, Hymenoptera, N-linked glycans, venom allergy, *Vespula* species, yellow jacket

Hyaluronidases are glycoside hydrolases cleaving β -1,4-glycosidic bonds between N-acetylglucosamine and D-glucuronic acid of hyaluronic acid and thereby act as spreading factors in Hymenoptera and other animal venoms.¹ Hyaluronidases have long been recognized as important allergens in honeybee venom,²⁻⁴ as well as yellow jacket venom, in which hyaluronidase represents one of 3 major allergens together with antigen 5 and phospholipase A (PLA) 1.^{5,6}

The hyaluronidases are the phylogenetically most strongly conserved Hymenoptera allergens. Sequence homologies between *Vespula* and *Dolichovespula* species hyaluronidases are 90% or greater, whereas those for antigens 5 and PLA₁ are only around 60% to 65%. In agreement with this, immunologic cross-reactivity between different vespid genera is strong with hyaluronidases but more restricted with antigens 5 and PLA₁.^{5,7,8} There is still significant similarity of vespid hyaluronidases with the hyaluronidase from honeybee venom (Api m 2), which shows around 50% sequence homology with the vespid homologs Ves v 2, Ves g 2, and Dol m 2.⁹ In concordance with this, hyaluronidases have been identified in inhibition studies using patients' sera as the most important cross-reactive allergens in yellow jacket and honeybee venom.^{6,10} Additional evidence for cross-reactivity comes from animal studies on hyaluronidase-specific murine IgG and T cells.¹¹ Thus double sensitization to yellow jacket and honeybee venom, which is encountered in up to greater than 50% of patients with insect hypersensitivity,¹²⁻¹⁶ has been considered to result from cross-reactivity between the venom hyaluronidases in many cases.

However, more recent studies revealed that much of the cross-reactivity between yellow jacket and honeybee venom is not due to protein cross-reactivity but is caused by cross-reactive N-glycans (cross-reactive carbohydrate determinants [CCDs]).^{14,15,17,18} The clinical relevance of CCD-specific IgE antibodies is believed to be very low, even if the reasons for this are not yet completely understood.¹⁹⁻²¹ Accordingly, a substantial number of positive test results in subjects with insect allergy must be considered false-positive. Using Western blot inhibition, we and others previously demonstrated a pivotal role of hyaluronidases in these CCD-mediated cross-reactions.^{15,17} Actually, venom hyaluronidases have been long known as glycoproteins,^{3,22} and their N-glycans have been characterized by means of HPLC and mass spectrometry to contain α 1,3-fucose as the key structure of allergenic N-glycans.²³⁻²⁵ In yellow jacket venom hyaluronidase represents the by far most important glycoallergen.^{17,25,26} Only small amounts of CCDs can be localized to

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Abbreviations used

CCD: Cross-reactive carbohydrate determinant
 DPP: Dipeptidylpeptidase IV
 PLA: Phospholipase A
 YJ-HYA: Yellow jacket hyaluronidase

dipeptidylpeptidase IV (DPP),^{26,27} and PLA₁ seems to be glycosylated in *Vespula squamosa* but not in other *Vespula* species.²⁶ Hyaluronidase is also a main glycoprotein in honeybee venom, although, different from yellow jacket venom, it contains quite a few additional glycoallergens, including PLA₂, acid phosphatase, CUB-serine protease, and DPP.²⁷ All these glycoproteins are strongly bound by CCD-positive human sera, as well as CCD-specific rabbit IgG.¹⁷

In light of these recent insights, the importance of hyaluronidase in yellow jacket allergy turns out to be uncertain. Much of the reactivity with this allergen described in previous studies might have been directed to CCDs but not to the peptide itself. Moreover, the observed cross-reactivity with honeybee hyaluronidase might primarily depend on CCDs and might not be related to shared peptide epitopes. In fact, although identical folding patterns have been verified by means of protein crystallization for Ves v 2 and Api m 2, the 2 allergens show significant differences in surface topology and charge distribution outside the active center.^{28,29} Cross-reactivity between the proteins has therefore been called into question.²⁸

This study was undertaken to critically reassess the importance of hyaluronidase in yellow jacket venom allergy by discriminating between IgE binding to peptide versus carbohydrate epitopes in a large number of patients with yellow jacket hypersensitivity. In addition, we studied the potential protein cross-reactivity between yellow jacket and honeybee hyaluronidase.

METHODS**Hymenoptera venoms**

Lyophilized pure yellow jacket venom sac extracts (mixture of *Vespula vulgaris* and *Vespula germanica*) were purchased from Vespa (Vespa Laboratories, Spring Mills, Pa). According to the manufacturer, the amount of antigen 5, PLA₁, and hyaluronidase in the venom preparation was 0.013, 0.041, and 0.010 µg/mg of total protein, respectively. Lyophilized whole bee venom was purchased from Nectarcorp (Sophia, Bulgaria).

Patients

Serum samples from 136 patients (73 male and 63 female patients; median age, 42 years; age range, 6–81 years) with a systemic reaction of varying severity (Mueller grade I–IV) after a vespid sting were included. In the majority of patients (97/136), reactions occurred after a sting from a yellow jacket (*Vespula* species), although in rare cases a *Dolichovespula* species might have been the causative insect. Twelve of 136 patients claimed a European hornet (*Vespa crabro*) as the culprit insect. In 27 of 136 cases, a yellow jacket was not identified with certainty as the responsible insect, but according to venom skin testing, yellow jacket allergy was very likely. All patients had a positive CAP result (Phadia Diagnostics, Uppsala, Sweden) to *Vespula* venom of at least 0.7 kU/L (class ≥2). Patients were grouped on the basis of CAP testing into yellow jacket single-positive (n = 31) and yellow jacket-honeybee double-positive (n = 105) cohorts. In addition, double-positive sera from 20 patients with honeybee venom allergy were studied.

Western blots with whole venom

Yellow jacket and honeybee venom were separated by means of SDS-PAGE (13.5% resolving and 5.7% stacking gel, 6 µg of protein per lane) under reducing conditions by using dithiothreitol and heat for *Vespula* venom and 2-mercaptoethanol and heat for honeybee venom. Pretrials excluded loss of IgE epitopes on YJ-HYA or other yellow jacket allergens by means of protein reduction, but reduced YJ-HYA (migrating at 43 kd) was more strongly bound by IgE and better delimited from PLA₁ than nonreduced YJ-HYA (migrating at 38 kd; see Fig E1 in this article's Online Repository at www.jacionline.org). Separated proteins were blotted onto nitrocellulose, and the membranes were cut into strips of 4 mm in width and blocked with PBS buffer (50 mmol/L sodium phosphate [pH 7.5], 0.5% Tween 20, and 0.05% NaN₃) containing 0.5% BSA at room temperature for 1 hour. Each strip was incubated overnight with 1 mL of serum (diluted 1:5 to 1:15) at 4°C with continuous shaking. After washing twice with PBS buffer, iodine 125-labeled rabbit anti-human IgE (Phadia) diluted to achieve 200,000 cpm per strip was added. After overnight incubation at 20°C, washed and dried strips were exposed to a high-performance autoradiographic film (Hyperfilm MP, Amersham, England) at –70 °C for 5 days.

Western blot inhibition

For inhibition, sera were preincubated with MUXF-BSA (5 µg/mL) to uncover IgE binding to carbohydrate epitopes and subsequently with a mixture of MUXF-BSA plus purified Api m 2 (5 µg/mL each) to reveal protein-specific cross-reactivity between yellow jacket and honeybee hyaluronidase.

MUXF-BSA, used as the main CCD inhibitor in this study, was synthesized by coupling purified bromelain glycopeptides to BSA, as described previously.³⁰ A final MUXF-BSA concentration of 5 µg/mL was found in pilot tests to absorb CCD-specific IgE effectively, even in samples with very high levels of anti-CCD IgE. Inhibitions were also performed with MMF3F6-BSA in some sera to evaluate whether MUXF glycans sufficed to absorb all specificities of anti-CCD IgE. This neoglycoprotein was prepared with glycopeptides from honeybee PLA₂, which contains the more insect-typical glycan MMF3F6.^{23,30} For inhibition, MMF3F6-BSA was mixed with MUXF-BSA (5 µg/mL each), and the inhibition patterns were compared with those from MUXF-BSA alone. Api m 2 was purified from honeybee venom, as previously described.²³

In some selected sera, reciprocal inhibition was carried out with blotted honeybee venom as the solid phase. Inhibition was performed with MUXF-BSA, MUXF-BSA plus Api m 2, and crude yellow jacket venom (30 µg/mL).

Screening for anti-CCD IgE by bromelain CAP

CAP to bromelain (k202) was performed in 100 sera randomly picked out from the 136 sera included in the study.

RESULTS**Single-positive sera from patients with yellow jacket allergy**

Among the 31 sera tested, only 1 showed clear reactivity with YJ-HYA (approximately 43 kd) in Western blots (Fig 1, A). IgE binding of this serum to hyaluronidase was not inhibited by MUXF-BSA and Api m 2 (data not shown), suggesting specific reactivity with the YJ-HYA protein. Antigen 5 (approximately 24 kd) and PLA₁ (approximately 33 kd) were each bound by 29 (94%) of the 31 sera, with 27 (87%) of 31 sera reacting to both allergens.

Double-positive sera from patients with yellow jacket allergy

Of 105 double-positive sera tested, as much as 87% (91/105) bound to YJ-HYA (occasionally appearing as a double band;

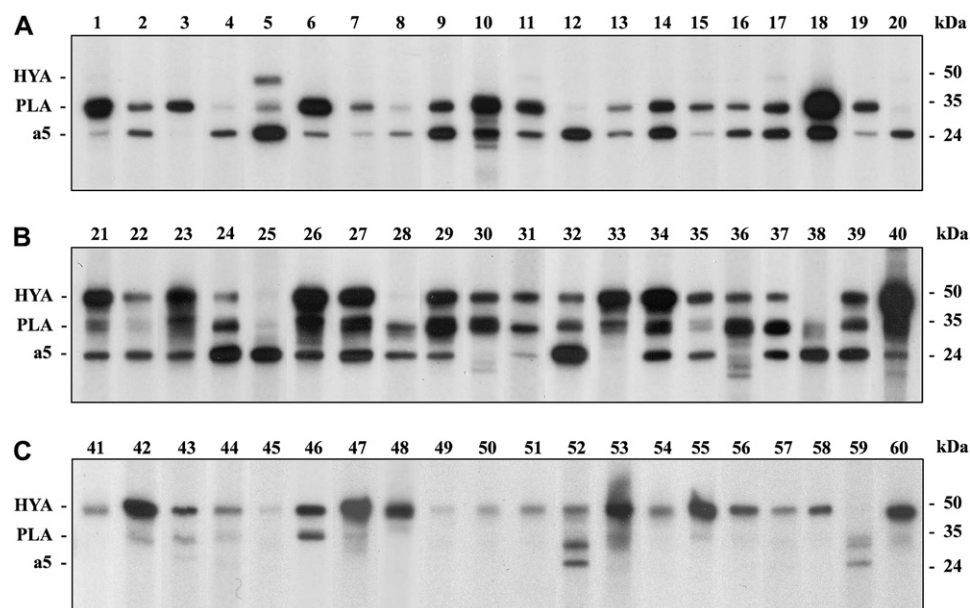


FIG 1. IgE binding to blotted *Vesputa* species venom. Twenty sera are shown from each group to illustrate the different binding patterns. **A**, Single-positive sera from patients with yellow jacket venom allergy. **B**, Double-positive sera from patients with yellow jacket venom allergy. **C**, Double-positive sera from patients with honeybee venom allergy. HYA, Hyaluronidase; a5, antigen 5.

Fig 1, B). Eighty-nine percent (93/105) of the sera bound to antigen 5, and 90% (95/105) bound to PLA₁. Eighty-seven of 105 sera reacted to both antigen 5 and PLA₁, and 101 of 105 sera reacted to at least one of the 2 allergens.

Immunoblot inhibition was carried out in 83 of 91 sera positive for the YJ-HYA band. After serum preincubation with MUXF-BSA, IgE binding to hyaluronidase was completely abolished in 54 (65%) of 83 sera, indicating reactivity with CCDs alone; partially abolished in 22 (27%) of 83 sera, indicating reactivity with CCDs and peptide epitopes (predominant reactivity with CCDs in 14/22); and unchanged in 7 (8%) of 83 sera, indicating restricted reactivity with peptide epitopes (**Fig 2, A-C**). In sera with high anti-CCD IgE levels, the 33-kD band (PLA₁) was often detected as a double band, with the second band situated immediately above the PLA major band (see **Fig 2, A**, part 1; **Fig 2, B**, part 1; **Fig 2, C**, part 1; and **Fig 3**, part 5). Because antibody binding to this band was always completely abolished by MUXF-BSA, this peptide possibly represents degradation products of hyaluronidase.

Altogether, 29 sera (22 + 7) specifically reacted with the YJ-HYA protein, according to inhibition with MUXF-BSA. When preincubated with Api m 2 plus MUXF-BSA, the peptide-specific reactivity with YJ-HYA was fully or at least partially inhibited in 14 (48%) of 29 sera, indicating protein-specific cross-reactivity with Api m 2 (**Fig 2, B and C**).

Reverse inhibition experiments were carried out with selected sera on honeybee venom blots to further explore the protein cross-reactivity with Api m 2, as exemplified in **Fig 3**. Patients 1 and 2 show protein-specific reciprocal cross-reactivity between YJ-HYA and Api m 2, which is consistent with the view of primary sensitization to YJ-HYA and cross-sensitization to Api m 2. In patients 3 and 4 specific binding to the YJ-HYA peptide was not cross-inhibited by Api m 2 because these sera bound with Api m 2 only through CCDs. **Fig 3**, part 5, shows true double

sensitization with primary sensitization to Api m 2. Here Api m 2 inhibited completely IgE binding to YJ-HYA but not *vice versa*.

Double-positive sera from patients with honeybee allergy

Double-positive sera from patients with honeybee allergy bound in yellow jacket venom most often to hyaluronidase (19/20; **Fig 1, C**). According to inhibition with MUXF-BSA, this was due to CCDs alone in 76% (13/17; **Fig 2, D**, parts 1 and 2, and **Fig 4**, part 1), whereas 4 of 17 sera reacted with peptide epitopes (**Fig 2, D**, part 3, and **Fig 4**, parts 2 and 3). Clear-cut binding to PLA₁ and antigen 5 (indicating true double sensitization) was seen in 6 and 3 samples, respectively. Altogether, nearly half of the sera reacted with yellow jacket venom only through carbohydrates.

Reciprocal immunoblot inhibition with honeybee venom as the solid phase was carried out in selected patients. **Fig 4** shows representative immunoblots and inhibition patterns from 3 patients with IgE against the Api m 2 peptide.

Inhibition experiments with MMF3F6 glycans

Inhibition of IgE binding to YJ-HYA by Api m 2 plus MUXF-BSA but not by MUXF-BSA alone was considered indicative of specific cross-reactivity through shared peptide epitopes. Because in rare cases serum IgE antibodies might specifically react with fucosylated N-glycans bearing 2 distal mannose molecules (as common in Hymenoptera proteins, including Api m 2) but not with glycans bearing just 1 mannose (as in MUXF), we performed additional inhibition experiments using MMF3F6 glycans obtained from whole honeybee venom. As exemplified in **Fig 5**, no additional inhibition compared with that seen with MUXF-BSA alone was obtained by MMF3F6 glycans, confirming that cross-inhibition by Api m 2 is due to shared peptide epitopes.

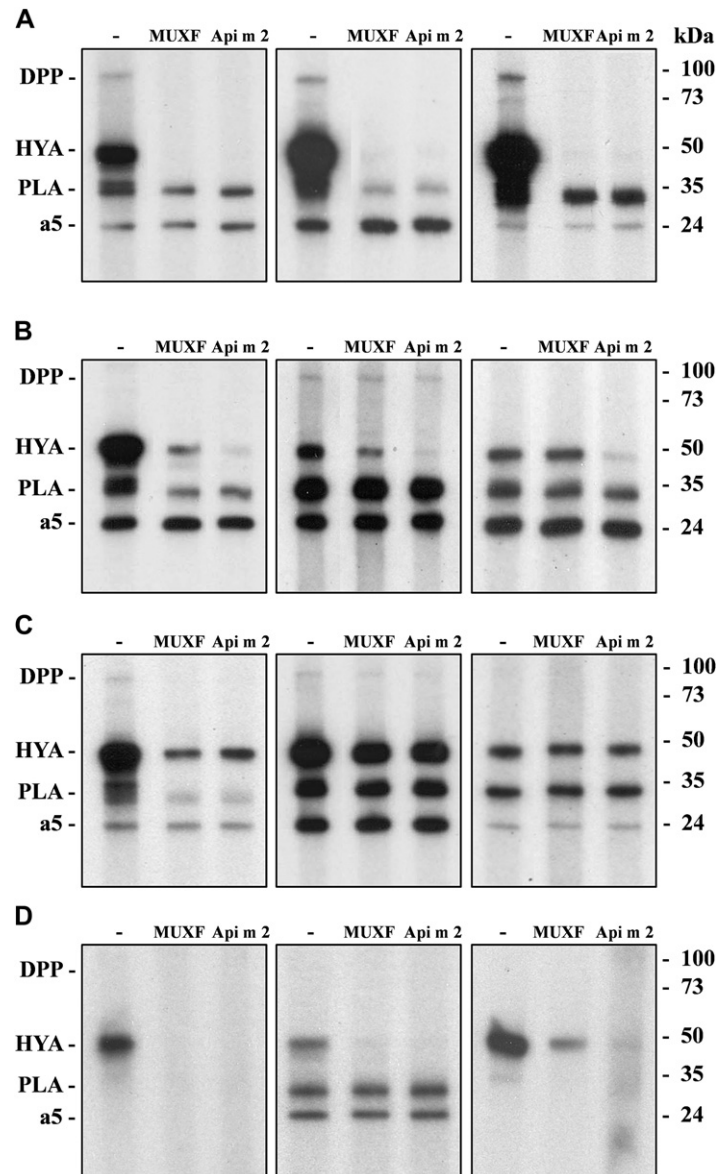


FIG 2. CCD- and protein-specific inhibition of IgE binding to *Vesputa* species venom in double-positive sera from 12 patients with yellow jacket (**A-C**) or honeybee venom allergy (**D**). **Fig 2, A**, Sera binding to hyaluronidase (HYA) CCDs only. **Fig 2, B and C**, Sera containing HYA peptide-specific IgE with (**Fig 2, B**) and without (**Fig 2, C**) cross-inhibition by Api m 2. **Fig 2, D**, Patients with honeybee venom allergy binding with HYA CCDs (sera 1-2) or CCDs plus protein (serum 3). —, Noninhibited serum; MUXF, inhibition with MUXF-BSA; Api m 2, inhibition with Api m 2 plus MUXF-BSA; a5, antigen 5.

IgE binding to high-molecular-weight *Vesputa* allergens

Some sera bound to a high-molecular-weight allergen of approximately 100 kd, presumably representing DPP (Ves v 3), a glycoallergen previously described as V mac 1.⁶ Twenty-six percent (23/89) of double-positive sera bound to DPP, but binding intensity was weak throughout. According to inhibition with MUXF-BSA, IgE was directed against CCDs in 17 of 23 sera (**Fig 2, A**) and in only 6 sera against the DPP peptide (eg, **Fig 2, B**, part 2).

Screening for anti-CCD IgE by bromelain CAP

Among 100 sera investigated, 88 were identified as CCD-positive according to Western blot inhibition with MUXF-BSA.

Only 73% (64/88) of them were positive at least at class 1 to bromelain (range, 0.35-82.5 kU/L). Sera testing negative were mainly those with only moderate binding to YJ-HYA CCDs in the Western blot.

DISCUSSION

Hyaluronidase has been considered thus far a major allergen in yellow jacket venom allergy, but this study indicates that it is only a minor allergen. Although nearly 90% of double-positive sera bound with hyaluronidase in Western blots, the reactivity was directed to CCDs in the majority of cases. Among yellow jacket venom single-positive sera, IgE to hyaluronidase appears to be uncommon. Assuming an overall prevalence of double positivity

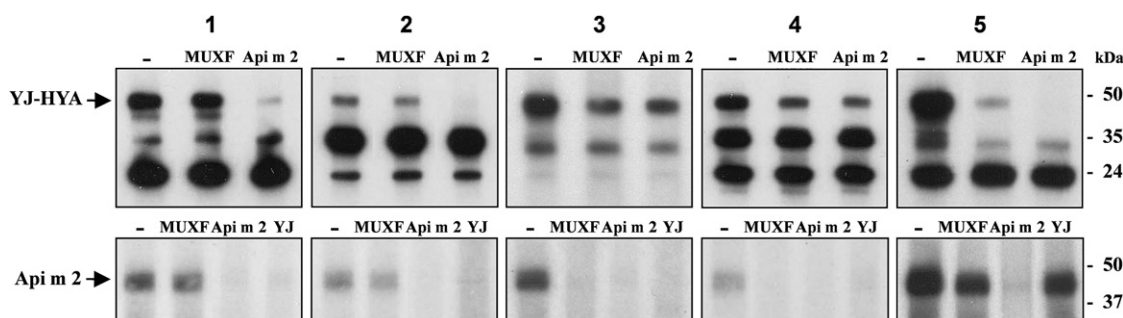


FIG 3. Inhibition of IgE binding to *Vesputa* species and honeybee venom hyaluronidases in double-positive sera from patients with yellow jacket allergy with IgE against the YJ-HYA peptide. 1 and 2, Reciprocal protein-specific cross-reactivity between YJ-HYA and Api m 2; 3 and 4, sera binding with Api m 2 only through CCDs; 5, patient with primary sensitization to Api m 2. —, Noninhibited serum; MUXF, inhibition with MUXF-BSA; Api m 2, inhibition with Api m 2 plus MUXF-BSA.

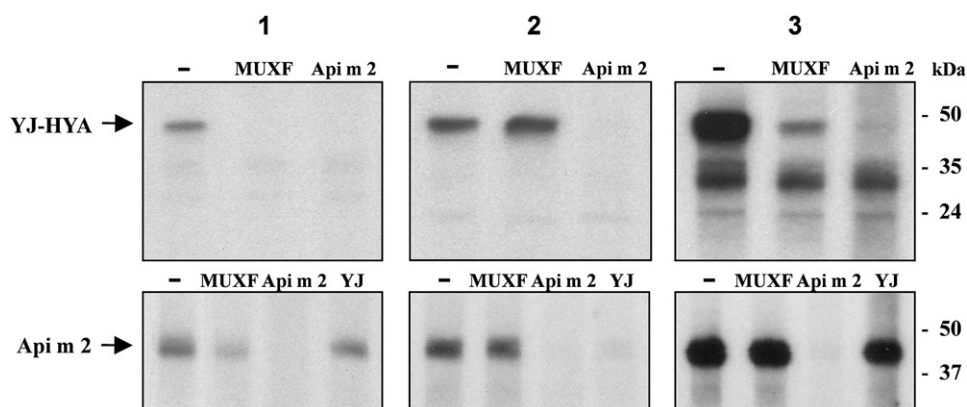


FIG 4. Inhibition of IgE binding to *Vesputa* species and honeybee venom hyaluronidases in double-positive sera from 3 patients with honeybee venom allergy. 1, Reactivity with YJ-HYA CCDs only; 2 and 3, IgE binding to HYA peptide epitopes with reciprocal (part 2) or one-sided (part 3) cross-inhibition between YJ-HYA and Api m 2. —, Noninhibited serum; MUXF, inhibition with MUXF-BSA; Api m 2, inhibition with Api m 2 plus MUXF-BSA; YJ, inhibition with yellow jacket venom.

of 20% to 40% within the population with insect allergy, we might calculate from our results that hyaluronidase is a relevant protein allergen for not more than 10% to 15% of patients with yellow jacket hypersensitivity. It seems reasonable to assume that antibody binding to hyaluronidases from other types of Hymenoptera, including paper wasps and bumblebees, is due to CCDs in many cases also. In particular, future studies will have to critically reinvestigate the role of hyaluronidase in the context of honeybee venom allergy, in which Api m 2 is considered the second-most important allergen after PLA₂.^{3,31}

The highly glycosylated DPP (Ves v 3) appears to be a true allergen for an even smaller minority of patients. IgE binding was mostly due to CCDs. Furthermore, binding intensities to this allergen were always weak. In contrast, antigen 5 and PLA₁ could be confirmed as major *Vesputa* species allergens because each of them was recognized by around 90% of double-positive and single-positive sera. This is in good agreement with a recent study reporting an 87% prevalence of IgE binding to recombinant antigen 5 in subjects with yellow jacket allergy.¹⁶

It must be pointed out that the classification of hyaluronidase as a minor allergen in this study is linked up with the assumption of a low clinical relevance of CCD-specific IgE antibodies. Although this view is supported by much of circumstantial and

experimental evidence from different fields of allergy, including pollen, food, and rubber latex allergy, it is still questioned by some *in vitro* observations, suggesting a potential clinical role of these antibodies, at least in some situations.^{19,20} What advocates for a low relevance of CCDs also within the scope of venom allergy is the fact that the immune response to the culprit venom is virtually always specific for protein epitopes, whereas binding to CCDs is typical for *in vitro* cross-reactivity.^{14,15,17,18} Furthermore, venom CCDs appear to elicit positive skin test responses only rarely.¹⁷ Surely, prospective sting challenge studies are required to conclusively prove or disprove the clinical relevance of anti-CCD IgE. For the time being, we appreciate judging these antibodies as clinically insignificant as a useful hypothesis, which considerably simplifies the diagnosis of venom allergy.

Another notable finding of our study is that virtually all patients with positive responses to hyaluronidase also have IgE against antigen 5, PLA₁, or both. Only 1 of the 136 patients studied had positive responses for the YJ-HYA peptide but not against antigen 5 or PLA₁. Notably, this patient was stung by a European hornet and not by a yellow jacket. We might speculate that his serum cross-reacted to YJ-HYA because of the higher similarity between the *Vesputa* and *Vespa* species homologs compared with the other allergens. Indeed, European hornet antigen 5 (Vesp c 5), the only

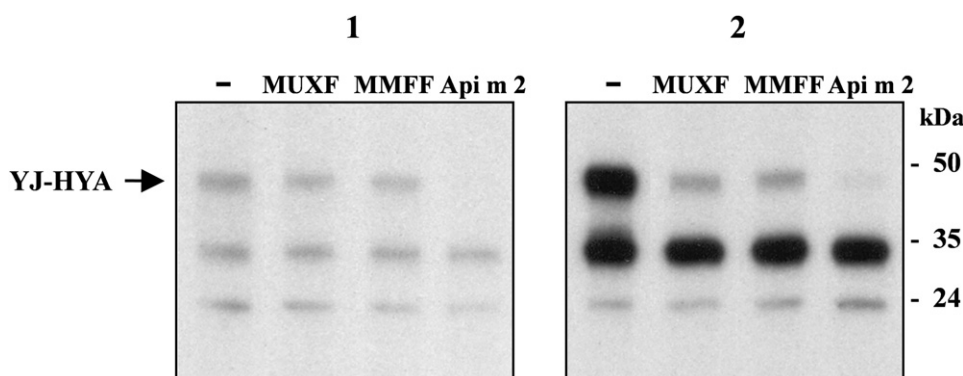


FIG 5. Inhibition of IgE binding to YJ-HYA by MUXF-BSA (MUXF; displaying N-glycans of the bromelain type) and by MMFF3F6-BSA (MMFF; displaying insect-typical MMFF glycans) in double-positive sera from 2 patients with putative IgE against the YJ-HYA peptide.

Vespa species allergen sequenced thus far, has only 67% to 69% sequence homology with its corresponding yellow jacket counterparts.³² With a view to component-resolved *in vitro* diagnosis, we might conclude from our data that antigen 5 and PLA₁ (preferably a mixture of both) will be sufficient to detect the vast majority of subjects with yellow jacket venom allergy.

The restricted relevance of YJ-HYA might be seen as a practical advantage because the expression of immunologically active nonglycosylated recombinant venom hyaluronidases in bacteria might be difficult.^{11,33} Production in insect cells facilitates proper protein folding but might lead to unwanted glycosylation patterns similar to the native protein.^{34,35} Although certain cell lines exhibit very low α -1,3-fucosyltransferase activity,³⁶ it is not yet clear to what extent the recombinant proteins display CCDs and how much this interferes with the correct diagnosis. The approach to component-resolved diagnosis might turn out more complex in subjects with honeybee venom allergy because of the higher number of potentially relevant allergens.^{31,37} However, in a recent study 96% of patients with honeybee venom allergy were given correct diagnoses by using recombinant PLA₂ (Api m 1) alone, suggesting that restricted sensitization to allergens other than Api m 1 is rare.¹⁶ Even if diagnosis with recombinant allergens proves slightly less sensitive, it has the big advantage of a much higher specificity.^{16,31} In particular, it would largely solve the problem of honeybee-yellow jacket double positivity because it makes possible the direct identification of true double sensitization through marker allergens specific for either honeybee or yellow jacket venom without interference by CCDs.¹⁶

Our study proves the existence of protein-specific cross-reactivity between the hyaluronidases from yellow jacket and honeybee venom, as proposed by early investigators.^{6,10} A possible contribution of carbohydrate-specific IgE with restricted reactivity to insect-typical MMF-glycans^{23,25} could be ruled out by inhibition experiments using the neoglycoprotein MMF3F6-BSA. However, although cross-reactivity between venom hyaluronidases is usually considered clinically important, little evidence exists for that. It is largely unknown whether systemic reactions to stings from both insects, which are yet rarely seen,^{13,18,38} root in true double sensitization or might also arise from cross-reactivity. A careful evaluation of such patients is required for a better insight into the potential significance of hyaluronidase cross-reactions.

We could confirm in the present study that reactivity with the second venom in double-positive sera is commonly due to CCDs

alone. Therefore *in vitro* screening for CCD-specific antibodies with the help of certain plant glycoproteins (eg, bromelain, horseradish peroxidase, and oilseed rape pollen) has been recommended as a simple routine measure providing information about individual sensitization patterns. Accordingly, a positive CCD test result would support irrelevant cross-reactivity through carbohydrates, whereas a negative test result would favor the presence of true double sensitization. It has been emphasized previously, however, that a positive CCD test result cannot reliably exclude true double sensitization because some sera might simultaneously contain IgE against CCDs, as well as unique peptide epitopes in both venoms.^{15,17} On the other hand, comparing the results from commercial bromelain CAP with the results from immunoblot inhibition in the present study suggests that neither negative bromelain CAP result consistently indicates true double sensitization because 27% of CCD-positive sera, mostly those with low anti-CCD-IgE levels, were not identified by bromelain CAP. Furthermore, a negative CCD test result cannot discern between true double sensitization and hyaluronidase-specific cross-reactivity. In the first case immunotherapy with both venoms might be indicated, whereas in the second case 1 venom should be sufficient. Hence although testing for CCDs provides some helpful clues about individual sensitization profiles, we must accept that its potential in predicting or excluding true double sensitization is limited.

The hyaluronidase from *Vespula vulgaris* (Ves v 2, Swiss-Prot P49370) was sequenced and cloned more than 10 years ago.⁹ It was discovered only later by means of MALDI-TOF mass spectrometry that the 43-kD hyaluronidase band seen in Western blots contains a comigrating second hyaluronidase protein different from Ves v 2.¹⁷ The new protein (UniProt Q5D7H4), currently designated Ves v 2.02 in the International Union of Immunological Societies database, has been fully sequenced and shows only 59% sequence homology with the "classical" YJ-HYA Ves v 2.01.^{25,26} Interestingly, there is about 10 times more Ves v 2.02 than Ves v 2.01 in the hyaluronidase band of electrophoretically separated yellow jacket venom, as judged from mass spectral intensities.²⁶ This is also the case in other *Vespula* species. It is currently unknown which one of the 2 hyaluronidases represents the relevant allergen. With respect to the cross-reactivity with Api m 2, both are possible candidates because they have comparable sequence homology with the bee protein (47% vs 44%). One might speculate that the by far more abundant Ves v 2.02 dominates as allergen, but a reliable estimation of the significance of the 2 proteins and their correlation with Api m 2 will require further investigations.

In conclusion, this study showed that 65% of sera binding to YJ-HYA react with its N-glycans only. In all, not more than 10% to 15% of patients with yellow jacket allergy are estimated to have IgE against the hyaluronidase protein, which therefore cannot be rated a major yellow jacket allergen any longer. We could provide new *in vitro* evidence for protein-specific cross-reactions between YJ-HYA and its homolog in honeybee venom, Api m 2, but the clinical relevance of this cross-reactivity is not well documented. The relative contribution of the 2 YJ-HYA homologs, Ves v 2.01 and Ves v 2.02, remains to be elucidated.

Key messages

- Hyaluronidase represents only a minor yellow jacket venom allergen and binds with human IgE mainly through its N-glycans.
- With a view to component-resolved diagnosis, antigen 5, together with PLA₁, will detect virtually all patients with yellow jacket allergy, whereas hyaluronidase is of minor interest.
- Cross-reactivity between the hyaluronidases from yellow jacket and honeybee venom is mainly induced by CCDs and less often because of shared peptide epitopes.
- The significance of the protein-associated cross-reactivity with regard to clinical hypersensitivity to both insects remains to be elucidated.

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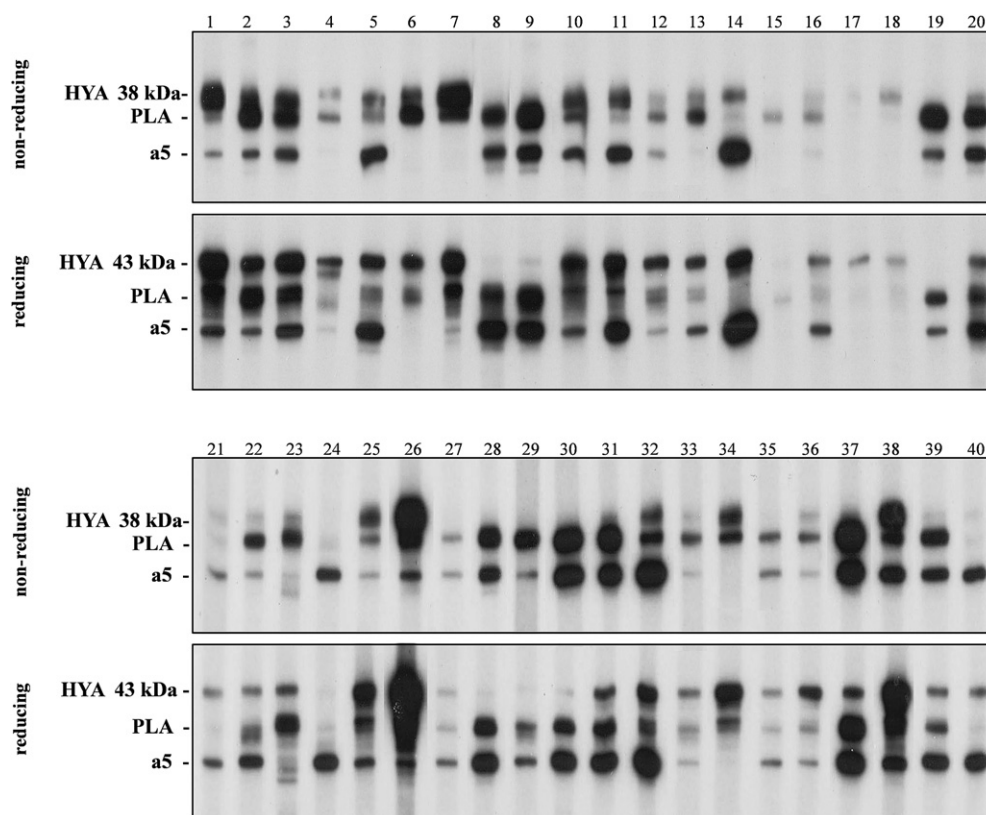


FIG E1. IgE binding of sera from 40 patients with yellow jacket allergy to *Vesputa* species venom separated by means of SDS-PAGE under nonreducing (*upper panel*) and reducing (*lower panel*) conditions. *HYA*, Hyaluronidase; *a5*, antigen 5.