

Immunotherapy with Commercial Venoms Is Efficacious for Anaphylactic Reactions to *Vespa orientalis* Stings

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Key Words

Immunotherapy • Venom allergy • *Vespa orientalis* • Yellow jacket

Abstract

Background: *Vespa orientalis* (VO) stings occasionally induce anaphylaxis. In the absence of commercial VO venom, allergists use commercial venoms for immunotherapy, despite having no indication regarding efficacy. We attempted to examine the effectiveness of immunotherapy with commercial venoms in patients with VO allergy and to identify the venom accountable for this effect. **Methods:** Patients who unequivocally identified VO as the culprit insect were administered venom immunotherapy with the commercial venoms available in Israel to which they had positive skin tests. Patients were also skin tested with VO venom sac extracts and, after reaching the maintenance dose, were sting challenged by a live insect. The allergenic components in the venom were determined by immunoblotting. **Results:** Twelve patients were recruited and, based on their skin test results, all were treated with yellow jacket (YJ) venom, either alone or combined with other venoms. All 8 patients who were sting challenged by VO demonstrated positive skin test responses to VO venom. Six of the stung patients tolerated

the sting challenge uneventfully. Two patients developed minimal transient symptoms that resolved spontaneously. SDS-PAGE with patient sera suggested cross-reactivity between VO and YJ venoms at molecular weights of 39–42 kDa. Using phospholipases, antigen 5 and hyaluronidase derived from several *Vespa*, *Dolichovespula* and *Vespula* species, hyaluronidase is possibly accountable for inducing the allergic reaction. **Conclusion:** In the absence of commercial VO venom the practice of treating patients allergic to this insect with available commercial venoms seems to be efficacious and YJ venom is probably responsible for this effect.

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Introduction

The old world hornets consisting of *Vespa orientalis* (VO) and *Vespa crabro* belong to the vespidae family, genus *Vespa*. Frequently found in the Mediterranean area, VO can also be found in southern Europe, Asia and northeast Africa as well as Madagascar [1, 2]. Data about anaphylactic reactions induced by VO sting are sparse.

Since a commercial extract of VO venom is not available, practicing allergists use other commercially available venoms for both diagnostic and therapeutic (immu-

notherapy) purposes in patients who have had a systemic reaction (SR) to VO stings. However, the efficacy of this policy has never been studied. To evaluate it, patients allergic to VO received VIT with commercial venoms. Patients were skin tested with self-prepared venom sac extract and after reaching the maintenance dose, they were challenged by a deliberate VO sting. The antigenic components in VO venom and their relationship to those of other Hymenoptera venoms were investigated by immunoblot and immunoblot inhibition techniques.

Methods

Patients and Skin Tests

All patients with an SR to insect sting who unequivocally recognized VO as the culprit insect were invited to participate. VO has a unique appearance with marked larger body size compared to other Hymenoptera, distinguished general brown color with two adjoining yellow stripes at the rear and elongated narrow wings. Only patients who correctly identified this insect on a demonstration tray containing the common Hymenoptera in Israel [honeybees, yellow jackets (YJ) and paper wasps (W; *Polistes* spp)] together with VO were enrolled. None of the patients had had an SR inflicted by a different insect other than VO. Patients were skin tested with bee venom (BV), YJ venom protein and W venom protein (ALK-Abello, Horsholm, Denmark). In some of the patients (those from Meir Medical Center) serum venom-specific IgE of the same venoms were measured with a commercial kit (AlaSTAT; DPC, Los Angeles, Calif., USA).

Patients were also skin-tested with self-prepared venom sac extracts from VO. Some of the patients were tested before commencement of venom immunotherapy (VIT) and some other patients who had already been receiving the maintenance dose were tested at this point.

Venom sacs of ether-anesthetized VO were gently exposed by pulling the stinger, and then removed and placed in a test tube. To extract venom, the sacs were homogenized in 500 µl of sterile saline by glass-to-glass homogenizer and then centrifuged (at 1,000 g for 15 min). Both the pellet and the supernatant were used. The pellet was resuspended in 1 ml saline and was rehomogenized by passing it through 23G and 30G syringe needles. Both the resuspended pellet and the supernatant were dialyzed overnight at 4°C in 500 ml of saline using a cellulose membrane dialysis tube (Sigma, catalogue No. D9777) which retains most proteins of molecular weight 12 kDa or heavier. The resultant venom-containing solutions were sterilized by filtration through a 0.20-µm syringe filter for further use. Protein concentrations of the filtered pellet and supernatant were 1.869 and 2.226 mg/ml, respectively.

Prick and intradermal skin tests were initially performed with both the pellet and the supernatant of the crushed venom sac extracts. In the first patients studied we noticed that the supernatants gave stronger positive skin reactions than the pellets. Therefore, we continued the study using only the supernatants. Skin prick tests were performed with 1:1000, 1:100 and 1:10 dilutions (w/v) of the filtered venom sac extract (protein concentrations of 2.23, 22.3 and 223 µg/ml, respectively). A higher concentration

was applied only if the previous, lower concentration resulted in a negative response. If all skin prick tests were negative, intradermal tests were similarly performed using the 1:100,000, 1:10,000 and 1:1000 dilutions (protein concentrations of 0.023, 0.223 and 2.23 µg/ml, respectively). A positive response was defined as a wheal ≥3 mm for prick tests or ≥5 mm for intradermal tests, compared with the negative saline control, accompanied by a flare reaction and a positive histamine response.

Rush (3-day build-up phase) or conventional (3- to 4-month build-up phase, at weekly intervals) VIT was administered with all the commercial venoms to which the patients demonstrated positive skin tests. If the patient was scheduled to receive several venoms they were administered concomitantly in conventional VIT, whereas in rush VIT different venoms were administered in separate courses at weekly intervals between each treatment course. Patients continued receiving a regular dose after reaching the maintenance dose of 100 µg of each of the venoms.

SDS-PAGE and Immunoblotting

Five micrograms of each venom (VO, BV, YJ and W) as well as 5 µg of the purified vespid phospholipases (*Vespula maculifrons*, *Vespula germanica* and *V. crabro*), 10 µg of the purified hyaluronidases (*V. germanica* and *V. crabro*) and 10 µg of the purified antigen 5's (*V. crabro* and *Dolichovespula maculata*) were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose filters. Blots were blocked for 2 h in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20] containing 5% skim milk, followed by overnight incubation at 4°C with the specific patient's serum diluted 1:20 with TBST. After washing 3 times with TBST, the blot was incubated for 1 h at room temperature with mouse anti-human IgE-HRP-conjugated secondary Ab (Southern Biotech., Birmingham, Ala., USA). Immunoreactive bands were visualized using the LAS-3000 imaging system (Fujifilm Corp., Tokyo, Japan). Integrated optical densities of the immunoreactive protein bands were measured as arbitrary units employing multi-gauge software (Fujifilm).

For immunoblot inhibition, the patient's serum (diluted 1:20 with TBST) was mixed with YJ protein venom to achieve a final protein concentration of 30 µg/ml. Bovine serum albumin (BSA) at a concentration of 30 µg/ml was used as negative control. The mixes were preincubated for 10 min at room temperature, followed by overnight incubation at 4°C with the membrane. Immunoblotting was performed as usual.

Purified vespid phospholipases, antigen 5's and hyaluronidases that had been prepared from hand-milked venoms of *V. maculifrons*, *V. germanica*, *V. crabro* and *D. maculata* were kindly donated by Dr. Donald R. Hoffman from the Brody School of Medicine at East Carolina University, Greenville, N.C., USA. These were preparations from 1980 to 1991 that were stored freeze-dried at -20°C.

Live Sting Challenge

After reaching the 100-µg maintenance dose of all venoms, patients were sting challenged, as previously described [3], with a live VO supplied by Jacob S. Ishay, PhD. Peak expiratory flow was measured before the challenge and in case of adverse symptoms, measurements were repeated. An informed consent approved by the Meir Medical Center ethical committee was signed by all patients before enrolling in the study.

Table 1. Patient characteristics

Patient No.	Gender	Age years	Severity of SR to field sting	Positive ST	Positive serum venom-specific IgE	Venoms administered for VIT	Type of VIT	Time between reaching MD and SC	Reaction to SC
1	M	20	4	YJ, W	YJ	YJ, W	Rush	40 months	none
2	F	45	3	YJ	ND	YJ	C	110 months	none
3	M	32	3	YJ, BV	ND	YJ, BV	C	70 months	3 transient urticarial lesions
4	M	44	1	YJ, W, BV	ND	YJ, W	C	32 months	none
5	M	45	2	YJ, W, BV	YJ, W, BV	YJ, W, BV	Rush	8 days	none
6	M	20	2	YJ, W	YJ	YJ, W	Rush	5 days	none
7	M	22	2	YJ, W	ND	YJ, W	C	50 months	none
8	M	17	3	YJ, W, BV	YJ	YJ, W, BV	Rush	6 days	mild cough
9	M	31	2	YJ, W, BV	YJ, BV	YJ, W, BV	ND	NC	NC
10	M	42	2	YJ, W	None	YJ, W	C	NC	NC
11	M	15	2	YJ, W	YJ	YJ, W	Rush	NC	NC
12	M	18	2	YJ	ND	YJ	Rush	NC	NC

C = Conventional; MD = maintenance dose; NC = not challenged; ND = not done; SC = sting challenge; ST = skin tests.

Results

Patients

Twelve patients (11 male, 1 female) were recruited. The average age was 29.3 ± 11.5 years (mean $\pm$ SD), range 15–45 years. The severity of their initial SR (Mueller criteria) to VO sting was grade 1 in 1 patient, grade 2 in 7 patients, grade 3 in 3 patients and grade 4 in 1 patient. Patient characteristics are presented in table 1.

Specific IgE to Commercial Venoms

All 12 patients had a positive skin test to YJ venom before initiation of VIT. One patient had a positive skin test to YJ venom only, whereas the others had positive skin tests to other venoms, including 6 patients to W venom, 4 to W venom and BV, and 1 patient to BV. Thus, the prevalence of positive skin tests for YJ venom was 100% (12 out of 12 patients), for W venom 83% (10 out of 12) and for BV 42% (5 out of 12). Serum venom-specific IgE was measured before the initiation of VIT in 7 patients. Six patients had a positive reaction to YJ venom. One patient also had a positive result to BV, 1 patient to BV and W venom, and 1 patient had negative results for all 3 venoms. Thus, the prevalence of serum venom-specific IgE for YJ venom was 86% (6 out of 7 patients), for BV 29% (2 out of 7) and for W venom 14% (1 out of 7).

Skin Tests with VO Venom Sac Extracts

Four nonallergic volunteers had borderline positive skin prick test results with the undiluted extracts and

negative results with the 1:10 dilution. Similarly, they had positive intradermal skin test results with the 1:100 dilution and negative results with the 1:1,000 dilution. Thus, a positive skin test result was defined as a positive response at $\leq 1:10$ dilution for skin prick test and at $\leq 1:1000$ dilution for intradermal skin test.

Skin tests with VO venom sac extracts were performed in 5 patients before the initiation of VIT and in 5 patients after they had been on the maintenance dose for an average of 53.2 ± 40.0 months (range 4–110). Eight out of 10 patients had positive skin prick tests with the 1:10 to 1:1000 dilutions. The other 2 patients who had negative skin prick tests had positive intradermal skin tests at the 1:1000 dilution. No signs of infection were notified after skin tests performed with self-prepared solutions.

Sting Challenge with VO

Sting challenge was performed in 8 patients. The challenge was performed 5–8 days after reaching the maintenance dose in 3 patients, after 4 months in 1 patient and after 32–110 months (mean 66) in 4 patients. Six patients tolerated the sting uneventfully. One patient developed three small urticarial lesions and another developed mild cough without wheezing and without significant change in peak expiratory flow. The symptoms in both patients subsided spontaneously. Considering the very mild, self-resolving urticarial lesions in the first patient and the presence of only mild subjective symptoms with no evident objective symptoms in the second patient, we continued administering the 100 μ g maintenance dose to

Table 2. Immunoblotting of venoms with patient sera drawn at different phases of VIT

	Molecular weight, kDa											
	Before commencement of VIT (n = 5)					While on maintenance dose of VIT (n = 4)						
	25	28	32	38	39–42	19	22	25	28	32	38	39–42
VO	1 (20)	4 (80)	3 (60)		5 (100)		1 (25)	2 (50)	1 (25)	3 (75)		4 (100)
YJ	1 (20)		1 (20)	1 (20)	5 (100)	1 (25)	1 (25)		2 (50)	2 (50)	3 (75)	1 (25)
BV					1 (20)		1 (25)					2 (50)
W				1 (20)								

Data are presented as number of patients with percentages in parentheses.

Table 3. VO and YJ venom immunoblot inhibition by preceding incubation of patient serum with YJ venom

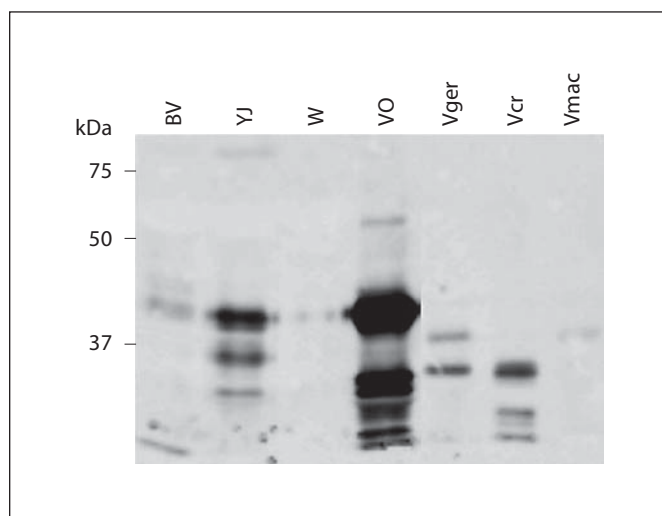
Ve-nom	Patient 1			Patient 2			Molecular weight kDa
	Serum only	Serum + YJ	Serum + BSA	Serum only	Serum + YJ	Serum + BSA	
VO	100	31.8	58.7	100	33.8	109.9	40
YJ	100	20.6	61.9	100	0	67.4	
VO	100	39.4	63.7	100	0	114.1	32
YJ	100	43.8	67.8	100	ND	ND	

Densitometry of 2 fractions with molecular weights of 40 and 32 kDa (numbers represent relative intensity of the staining expressed as percentage). ND = Not done.

these 2 patients. Two months after the first monitored challenge, 1 patient deliberately restung himself with VO in the field, and tolerated this second challenge well.

Immunoblotting

This was performed in 9 patients (before commencement of VIT in 5 patients and while on the maintenance dose in 4 patients). Sera from all 9 patients demonstrated several positive staining lines with VO and YJ venoms. Specifically, positive staining at molecular weights of 39–42 kDa with VO venom was demonstrated in all 9 patients, whereas with YJ venom, staining at these molecular weights could be shown in 6 patients (67%). Staining of YJ venom at these molecular weights was found in all patients before commencement of VIT (5 out of 5, 100%) and only in the minority of patients (1 out of 4, 25%) who had already been on the maintenance dose (table 2). Fewer patients demonstrated positive staining with other, lower molecular weight bands of both VO and YJ venoms. Positive staining was less common with bee and wasp venoms.

**Fig. 1.** A representative immunoblot staining of venoms and phospholipases from a single patient. Vcr = *V. crabro* phospholipase; Vger = *V. germanica* phospholipase; Vmac = *V. maculifrons* phospholipase; VO = VO venom; W = W venom; YJ = YJ venom.

Immunoblot inhibition was performed with sera of 2 patients, before commencement of VIT. Due to shortage of sera, tests for inhibition were performed once and not in duplicates. In both patients incubation of the patient's serum with YJ venom before immunoblot of VO venom significantly inhibited the staining of fractions with molecular weights of 40 and 32 kDa as compared to control preincubation with BSA (table 3). An unexpected and difficult-to-explain inhibitory effect of BSA ranging between 30 and 40% was noticed in some of the experiments but it was still notably smaller compared to the 60–100% inhibition induced by the venoms.

Immunostaining with purified phospholipases from *V. germanica*, *V. maculifrons* and *V. crabro* was performed in 8 patients. Many patients demonstrated positive stain-

Table 4. Immunoblotting of purified vespid phospholipases with patient sera drawn at different phases of VIT

	Molecular weight, kDa							
	before commencement of VIT (n = 5)				while on maintenance dose of VIT (n = 3)			
	20	26	37	42	22	28	32	37
<i>V. germanica</i>		1 (20)	1 (20)	1 (20)		1 (33)	1 (33)	1 (33)
<i>V. crabro</i>		1 (20)	1 (20)	2 (40)	1 (33)	2 (67)	1 (33)	
<i>V. maculifrons</i>	1 (20)	1 (20)						1 (33)

Data are presented as number of patients with percentages in parentheses.

ing with the phospholipases of *V. crabro* and *V. germanica* (75 and 62%, respectively), whereas some (25%) demonstrated positive staining with *V. maculifrons* phospholipase. Nevertheless, the staining was not restricted to any particular molecular weight and was rather equally distributed among various molecular weights ranging between 20 and 42 kDa. Immunoblotting results before commencement of VIT and while on the maintenance dose are shown in table 4. An example of immunoblotting with a single patient's serum is presented in figure 1.

Immunostaining of antigen 5 and hyaluronidase was performed with sera of 5 patients before commencement of VIT. Antigen 5 was derived from *D. maculata* and *V. crabro* venoms and hyaluronidase was derived from *V. germanica* and *V. crabro* venoms. No staining of antigen 5 was detected with any of the sera whereas both hyaluronidases were stained with all 5 sera. Staining at a molecular weight of 40–44 kDa was detected for both hyaluronidases in the sera of all 5 patients. Staining at a molecular weight of 34–37 kDa with hyaluronidase derived from *V. crabro* venom was detected in all patients as compared with that of hyaluronidase derived from *V. germanica* venom which was detected in 3 out of 5 patients.

Discussion

Despite the wide distribution of VO in Asia, Africa and southern Europe, a survey of the literature revealed only a single case report [4] of SR inflicted by VO, whereas other case reports have concentrated on the toxic, non-allergic morbidity, manifesting mostly as renal failure, which was provoked by multiple insect stings [5–10]. Reports on SRs including fatalities inflicted by the other old world hornet *V. crabro* are much more common [11–15] and it has even been shown that the relative risk of a life-

threatening reaction after a *V. crabro* sting was about three times higher than it was for honeybee or YJ sting [15]. Spread of *V. crabro* to the USA and to Australia has been reported [16, 17], but to the best of our knowledge, VO has been found only once west of Europe [18].

Initially, when the biochemical and immunologic properties of venoms from different *Vespa* and *Dolichovespula* species including VO were compared, it was concluded that immunotherapy with commercially available venoms of *Dolichovespula* should be adequate for most patient's sensitive to *V. crabro* but probably not for a significant proportion of those individuals primarily sensitive to VO [19]. This study did not examine YJ venom. However, another study [20] which also relied on the cross-reactivity between the two venoms claimed that commercially available YJ venom was appropriate for the treatment of *V. crabro* allergy. Nevertheless, this conclusion [20] was rather a supposition since the degree of protection provided by YJ-VIT has not been objectively confirmed in patients receiving this treatment. In another study, 2 patients allergic to *V. crabro* were indeed treated with YJ venom, but the efficacy of this approach has not been evaluated [21].

VO is widely distributed in Israel, but the actual incidence of SRs induced by its stings is not known. Still, it is well known among Israeli allergists that these reactions are not exceptional. That is why it was so important to confirm the effectiveness of the practical approach taken here and probably worldwide, i.e. administration of commercially available venoms to VO allergic patients. Therefore, recruitment for this study included only patients whose allergy to VO was confirmed by an unequivocal identification of the stinging insect and by the positive skin tests with VO venom. Indeed, after having received VIT with commercial venoms, although 2 patients had minimal transient symptoms following VO sting, practi-

cally all of them tolerated it well. The natural history of VO stings is unknown. Nevertheless, considering the anaphylactic nature of SRs occasionally induced by VO stings and the phylogenetic resemblance of different *Vespa* and *Vespula* species, conducting a placebo-controlled study in patients who have experienced anaphylactic reactions to VO stings seems unethical. Thus, despite the relatively small number of patients included here, this preliminary report supports the notion of the effectiveness of VIT with commercially available venoms in VO allergy.

All our 12 patients had positive skin tests to YJ venom. Most of them (83%) also had positive skin tests to W venom and less than half (42%) to BV. This is consistent with the high cross-reactivity between *Vespidae* species and the low cross-reactivity between *Apidae* and *Vespidae* which has already been acknowledged repeatedly in the past. Immunoblotting also established the presence of specific IgE against both YJ and VO venoms in all patients and immunoblot inhibition reaffirmed the cross-reactivity between the two venoms. However, the specific antigens that were recognized by the patients' sera were not identical for both venoms. Whereas positive staining with VO venom could be detected at molecular weights of 39–42 kDa in all patients both before commencement as well as after reaching the maintenance dose of VIT, this staining with YJ venom was detected in all patients before commencement of VIT but only in a minority of patients after reaching the maintenance dose. This difference may suggest that, as has already been described with other VIT protocols, the level of specific IgE directed against the administered venom tends to decline over time.

Considering the lack of well-established data on the cross-reactivity between VO venom and other venoms, before commencement of the present study we chose to include BV in the immunotherapy protocol despite the above-mentioned general low cross-reactivity between *Apidae* and *Vespidae*. In the light of our results this approach was probably unnecessary and VIT with YJ venom alone or with the addition of W venom should suffice to provide protection from future VO stings.

The common antigens in all vespids are hyaluronidase, phospholipase A1 and antigen 5 [22] with molecular weights of 38–39, 34 and 23 kDa, respectively [23]. The cross-reactivity of these antigens between different species of hymenoptera has been studied extensively [19, 24–27], especially by Hoffman et al. [26]. However, only a single study [19] has examined this issue in regard to VO. It found that although strong cross-reactivity was present between the venoms of VO and *V. crabro*, and between the venoms of *V. crabro* and both *Dolichovespula macu-*

late and *Dolichovespula arenaria*, VO venom had only a weak cross-reaction with the two *dolichovespulae*. Therefore, although it would theoretically be possible to speculate on the cross-reactivity of VO venom according to its cross-reactivity with *V. crabro* venom, such assumptions are not well substantiated. Indeed, Severino et al. [28] demonstrated the existence of cross-reactivity between *V. crabro* and YJ venoms. However, data about the role of hyaluronidase and *V. crabro* allergy are conflicting. Whereas one study showed that hyaluronidase was the major antigen in 3 out of 4 patients who were allergic to *V. crabro* [26], two other studies concluded that the role of hyaluronidase in YJ venom allergy was minute [29, 30]. We used immunoblot to explore whether sera of VO-allergic patients could detect the major vespid antigens. Antigen 5 was not detected by any of the sera. As for phospholipases, many of the patients demonstrated a positive staining, but it was not restricted to any particular molecular weight and was roughly equally distributed among various molecular weights. Although this finding could result from a partial decomposing of the phospholipases, which were 20–30 years old, it is still in accordance with a previous study that suggested that there were multiple antigenic determinants on the phospholipase molecule and that individuals responded to different determinants [25]. In contrast, hyaluronidases derived from both *V. crabro* and *V. germanica*, which were also as old as the phospholipases, were detected at a molecular weight of 40–44 kDa by all sera and at 34–37 kDa by the majority of the sera. This may suggest that hyaluronidase is the major antigenic component in VO venom. Conversely, it has also been shown in YJ allergy that many of the specific anti-hyaluronidase IgE antibodies are cross-reactive carbohydrates determinant-specific IgE antibodies that have very low clinical relevance [29, 30]. All in all, although our results suggest that hyaluronidase is probably the major antigen in VO venom allergy, this supposition should be evaluated cautiously considering the age of the purified antigens used.

In Israel the common hymenoptera include honeybees, YJs, W and VO. Accordingly, the commercial venoms used for diagnosis and immunotherapy include the above-mentioned venoms, except VO venom which is not available commercially. Since only VO and *V. crabro* belong to the *Vespinae* subfamily, theoretically, the closest venom to VO venom should be that of *V. crabro*. A commercial *V. crabro* venom is not available from our supplier and before commencement of the present study we were not aware about its availability from another source (Anallergo, Florence, Italy). It might be of great interest

to compare the diagnostic and therapeutic efficacy of this venom with that of the venoms we have used in our study, especially with YJ venom.

Based on the favorable outcome of sting challenges and supported by skin tests and immunoblot techniques we conclude that the common practice of treating patients who have anaphylactic reactions to VO with the commercially available venoms is effective. Although we have also used other commercial venoms we assume that YJ venom is primarily responsible for inducing this protective effect and suspect that hyaluronidase is the major allergenic constituent in VO venom. The small number of patients in the present study necessitates a larger study to ascertain whether this practice is appropriate. A larger

study may better define whether YJ venom alone or possibly commercial *V. crabro* venom might be sufficient for VIT of VO allergy, or whether further efforts are required to attain pure commercial VO venom for this purpose.

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