

Allergens in Hymenoptera venom XVI: Studies of the structures and cross-reactivities of vespid venom phospholipases

Donald R. Hoffman, Ph.D. Greenville, N. C.

Phospholipases (PLs) isolated from the venoms of three species of yellow jackets, white-faced hornets, European hornets, and paper wasps were studied by peptide mapping after limited enzyme hydrolyses and cyanogen bromide cleavage. Significant differences in the primary structures were observed. Cross-reactivities of vespid PLs were studied by precipitation reactions in gel with rabbit antibodies, immunoblots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels with rabbit antisera, and RAST inhibition with individual sera from vespid-reactive patients. The cross-reactivities of both human IgE antibodies and rabbit IgG antibodies were variable and not reciprocal between antigen and antibody, suggesting that there are multiple antigenic determinants on the PL molecules and that individuals respond to different determinants. No general patterns of cross-reactivity could be observed. (J ALLERGY CLIN IMMUNOL 78:337-43, 1986.)

Recent studies¹⁻⁹ have demonstrated that the venoms from vespid wasps all contain three major proteins, PL A₁ and B, antigen 5, and hyaluronidase, as well as several trace proteins. All three major proteins can be important allergens.⁷ One of the current problems in the clinical management of venom-allergic patients is the existence of multiple reactivity in a large fraction of patients.¹⁰⁻¹² Current recommendations and the general practice among allergists is to treat the multiply reactive patient with all the venoms that elicit positive reactions by either RAST or skin test. This is believed to be unnecessary in many cases in which the multiple reactivity is a result of antigenic cross-reactivity. In a few cases RAST-inhibition studies have been performed demonstrating that the primary reactivity of some patients can be determined¹²; however, this is an expensive and difficult procedure.

It has been suggested that some of the allergens of vespid venoms may demonstrate strong species or group-specific reactivities that could be used to determine a patient's primary sensitivity.⁹ The antigen 5s are significantly cross-reactive among *Vespula* (yellow jackets), *Dolichovespula* (hornets), and *Polistes*

Abbreviations used

PL:	Phospholipase
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Vm:	<i>Vespula maculifrons</i>
Vs:	<i>Vespula squamosa</i>
Vv:	<i>Vespula vidua</i>
Dm:	<i>Dolichovespula maculata</i>
Vcr:	<i>Vespa crabro</i>
Pex:	<i>Polistes exclamans</i>
TBS:	Tris-buffered saline, pH 7.4
TPCK:	L-1-tosylamide-2-phenylethyl chloromethyl ketone
MET:	Methionine
PITC:	Phenyl isothiocyanate

(paper wasps).^{8, 9} The hyaluronidases cross-react among the bees, *Vespula*, and *Dolichovespula*, but probably not with the *Polistes*.^{5, 8, 9} Vespid PLs are totally unlike bee PLs in both molecular weight and enzyme specificity.^{1, 2, 4} The vespid PLs appear in preliminary studies to exhibit significantly less cross-reactivity than the antigen 5s.^{8, 9}

In this study we examine the structure of vespid PLs from the three North American subgenera of *Vespula* yellow jackets, Dm, (white-faced hornet), Pex, (a paper wasp), and Vcr (European hornet) by limited hydrolysis peptide mapping. The cross-reactivities of the PLs were investigated by gel-diffusion experiments with rabbit antisera, immunoperoxidase-stained

From the Department of Pathology, East Carolina University School of Medicine, Greenville, N. C.

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Reprint requests: Donald R. Hoffman, Ph.D., Department of Pathology, East Carolina University School of Medicine, Greenville, NC 27834.

TABLE I. Amino acid compositions of vespid venom phospholipases

	Vm*	<i>V. germanica</i> ³	Vs*	<i>Dm1</i> *	<i>Dm2</i> *	<i>Dm1</i>	Pex*	<i>P. annularis</i> ⁴
ASP	27	27	31	28	26	32	29	35
GLU	31	32	27	26	25	29	23	21
SER	28	26	32	20	20	23	23	21
GLY	23	23	21	24	23	27	25	25
HIS	8	9	8	4	8	9	6	10
ARG	14	12	15	14	18	17	10	10
THR	23	24	22	14	20	21	19	17
ALA	20	21	19	18	19	20	21	26
PRO [†]	15	13	11	26	22	13	14	10
TYR	13	13	11	13	13	11	17	15
VAL	20	18	24	18	21	21	24	23
MET	3	4	3	3	4	4	4	2
CYS	10	12	11	7	7	10	7	13
ILE	22	22	22	24	19	17	17	17
LEU	20	22	18	25	18	19	24	24
PHE	13	14	13	15	17	15	16	15
LYS	24	24	26	26	24	30	33	30

ASP = aspartic acid; GLU = glutamic acid; SER = serine; GLY = glycine; HIS = histidine; ARG = arginine; THR = threonine; ALA = alanine; PRO = proline; TYR = tyrosine; VAL = valine; MET = methionine; CYS = cystine; ILE = isoleucine; LEU = leucine; PHE = phenylalanine; LYS = lysine.

*Determined by gas-phase hydrolysis and reversed-phase separation of PITC derivatives; average of duplicate determinations of two hydrolyses rounded to the nearest integer; standardized with bee venom phospholipase.

†An artifact peak in some cases overlaps proline.

nitrocellulose blots of SDS-PAGE, and RAST-inhibition studies by use of a panel of individual sera from allergic patients.

MATERIAL AND METHODS

Antigens

Pure venoms were obtained from individual vespids by milking with and without electrical shock as we have previously described.^{6, 13} Some of the venom was collected by removing the sacs from frozen insects and gently squeezing the venom from the sac down the sting shaft. This material is identical to naturally stung venom. The isolation of PLs from Vs, Dm, and Pex has been previously described.⁶ PLs from pure Vm venom, Vr venom, and Vcr venom were isolated by similar combinations of Sephadex G-75 gel filtration and high-performance cation exchange. All preparations were free of detectable antigen 5 and hyaluronidase; all demonstrated PL A and B activities.

Rabbit antisera

Antisera against purified venom components from Vm, Vs, Dm, and Pex were raised in New Zealand white rabbits as we previously described.⁵

Peptide maps

Purified proteins were heated at 100° in SDS-PAGE treatment buffer containing dithiothreitol at a concentration of 100 µg/ml and then digested for 1 hour with 0.75 µg of

enzyme per 50 µl of substrate. The enzymes used were α-chymotrypsin, TPCK-trypsin, and staphylococcal protease V8, all purchased from Worthington Diagnostic Systems, Freehold, N. J. After digestion the specimens were applied to a 15% total acrylamide concentration SDS-PAGE gel and electrophoresed. After fixation the gels were stained with a silver stain (Bio-Rad Laboratories, Richmond, Calif.). It was necessary to recycle gels through the silver and developer for optimal results. The digestion method was modified from that of Cleveland et al.¹⁴ CNBr digests were prepared by the method of Lam and Kasper,¹⁵ and the gels were processed similarly to the enzyme digests.

Immunoblot

SDS-PAGE minigels were run in a Hoefer Scientific (San Francisco, Calif.) Mighty Small apparatus with 0.75 mm spacers. The gel system was that previously described.⁵ Prestained molecular weight standards from Bethesda Research Laboratories (Gaithersburg, Md.) were used to monitor the electrophoresis and transfer, 0.5 µg of venom protein was applied to each well, and SDS-PAGE was performed at 20 mA per gel. After completion of electrophoresis, proteins were electrophoretically transferred to nitrocellulose membrane (Whatman). The transfer was performed by the method of Towbin et al.¹⁶ with 0.1% SDS added to the buffer. The membrane was then blocked with 5% bovine serum albumin for 1 hour at 37° and incubated overnight with 20 ml of a 1:100 dilution of rabbit antiserum in 0.02 mol/L of Tris-HCl, pH 7.4, and 0.15 mol/L of NaCl

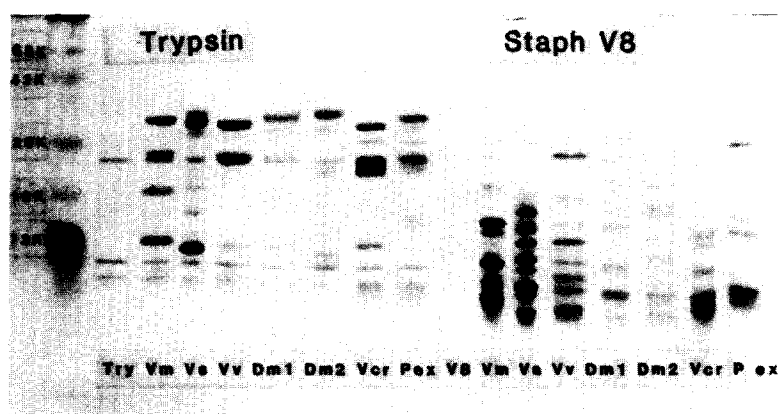


FIG. 1. Peptide maps generated by limited hydrolysis of purified vespid PLs by TPCK-trypsin on the *left* and staphylococcal V8 protease on the *right*. The far *left-hand* track contains molecular weight markers. *TRY* and *V8* indicate the patterns of the enzymes without substrate. The digest of 1 μ g of protein was applied to each well.

containing 1% gelatin. The blot was washed once in water and twice for 10 minutes in 100 ml of Tris-NaCl containing 0.05% Tween 20. The blot was then incubated with 50 ml of a 1:3000 dilution of goat antirabbit IgG horseradish peroxidase (Bio-Rad Laboratories) in 1% gelatin TBS for an hour, washed in water and twice in Tween-TBS, and then developed in a solution of 100 ml of TBS containing 60 mg of 4-chloro-1-naphthol in 20 ml of methanol and 0.015% hydrogen peroxide. Reactions were quantitated by reflectance densitometry.

RAST disks

RAST disks were prepared with limiting amounts of antigen as we previously described.⁷ Those used in the RAST inhibition were from the same lots as those used in previous studies.^{7, 8}

RAST inhibition

Inhibition experiments were performed as we previously described in detail.¹² All tests were performed at four or six concentrations of inhibitor. All sera elicited from 6% to 30% specific binding with no inhibitor added.

Patient sera

Sera were from untreated patients who experienced recent systemic reactions from vespid stings. Many of the sera have been used in other studies from our laboratory.^{5, 7, 8, 12, 17, 18}

Amino acid analysis

Microgram amounts of protein were hydrolyzed by gas phase hydrochloric acid containing 1% phenol. The amino acids were derivatized with phenylisothiocyanate and separated and quantitated on a reversed-phase high-performance chromatography column (Pico-Tag, Waters Associates, Milford, Mass.). The procedure was slightly modified from Bidlingmeyer et al.¹⁹ All results were calculated from the results of two hydrolyses, each measured in duplicate.

Compositions were corrected by use of a bee venom PL standard, and results are expressed rounded to the nearest integer.

RESULTS

PLs were purified from the venoms of Vm, Vs, Vv, both isozymes from Dm, Vcr, and Pex. All were similar in molecular weight to those described in the literature,^{1, 3-6} and all had both PL A and B activities with egg yolk lecithin as substrate. Amino acid compositions have been reported by King et al. for several of the PLs^{1, 3, 4, 9}; compositions measured in our laboratory by reversed-phase separation of PITC derivatives¹⁹ were similar, as can be observed in Table I. We also observed significant differences in the amino acid compositions of the PLs from different insects.

Limited proteolytic digests were prepared from each of the PLs by treatment of reduced SDS-denatured PL with either chymotrypsin, TPCK-trypsin, or staphylococcal V8 protease.¹⁴ CNBr digests were also prepared.¹⁵ The digests were run on 15% total acrylamide concentration SDS-polyacrylamide gels to generate peptide maps. Peptides were visualized with silver stain. The peptide maps of the seven PLs digested with TPCK-trypsin and V8 protease are illustrated in Fig. 1. The PLs were not very susceptible to trypsin digestion under the conditions used, but several similarities can be observed. The PLs generate fragments of 21,000 to 24,000 MW (about the same size as antigen 5) as well as some fragments of about 12,000 MW. The V8 protease that cleaves primarily at the carboxyl terminal of glutamic acid generates a series of fragments from each PL, demonstrating only limited similarities among the patterns from each spe-

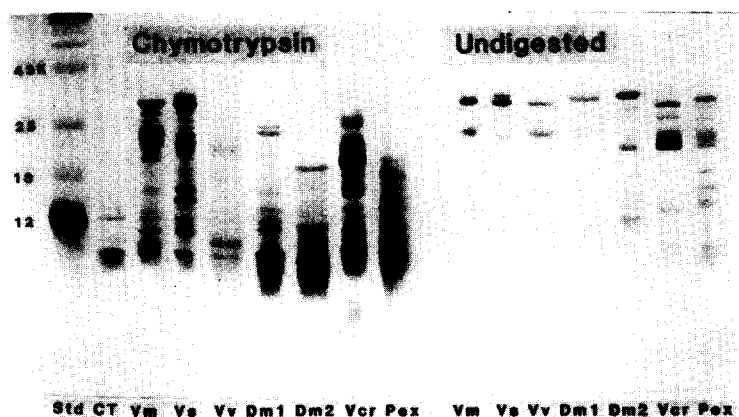


FIG. 2. Peptide maps of purified vespid PLs subjected to limited hydrolysis with chymotrypsin. Molecular weight markers are in the far left-hand track. CT indicates the pattern of chymotrypsin alone. The digest from 1 µg of protein was applied to each well. The right-hand side of the figure demonstrates the patterns of undigested PLs. The fragments result from natural hydrolysis and are released by the reducing agent in the sample buffer. Vcr PL was purified from commercial sac extract that contained proteases and not from pure venom.

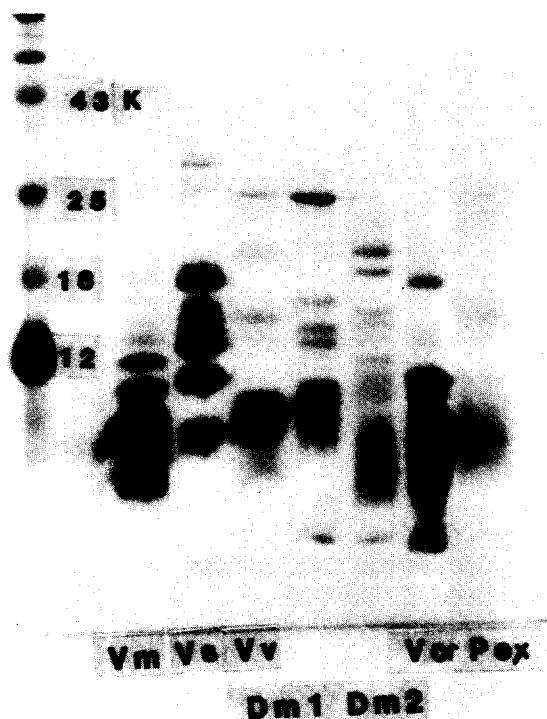


FIG. 3. Peptide maps produced by hydrolysis of purified vespid venom PLs with CNBr. Track 1 contains molecular weight markers. Sample loadings were the digests of 4 µg of protein.

cies. The chymotryptic maps illustrated in Fig. 2 have almost no similarities among the digestion patterns. The CNBr digests are illustrated in Fig. 3. CNBr cleaves proteins at MET residues, and the acidic conditions used may also produce some cleavages at

aspartic acid residues. The fragmentation patterns among the seven PLs again appear to be significantly different. This is not unexpected because the number of MET residues has been reported to vary from two to four among the PLs.⁹ We found similar variation of the MET content among our PL preparations. Since MET is usually a highly conserved amino acid in related sequences, this suggests that the vespid PLs demonstrate significant evolutionary divergence.

Rabbit antisera were raised against the purified PLs from Vm, Vs, Dm isozymes 1 and 2, and Pex. The results of double-diffusion precipitation reactions of four of the antisera with the five antigens are illustrated in Fig. 4. All the antisera reacted most strongly with the immunizing antigens. The Dm isozymes were partially cross-reactive. Weak cross-reactions were observed between Vm and Vs PL, Pex and Vm PL, and between Dm isozyme 2 and *Vespula* PLs.

The rabbit antisera were further tested with SDS-PAGE immunoblots, where the denatured venom proteins were separated by molecular weight by electrophoresis, then transferred to nitrocellulose, and reacted with rabbit antibody, and the antibody binding was detected with horseradish peroxidase-coupled anti-rabbit IgG. The results of these experiments are illustrated in Fig. 5. The two antisera raised against Vm PL reacted most strongly with homologous PL and demonstrated significant cross-reactivity with both of the other *Vespula* species. Serum B3 reacted weakly with Dm and Pex but not with *Vespa* PL, whereas serum B4 cross-reacted with all of the vespid PLs. The serum raised against Vs PL cross-reacted

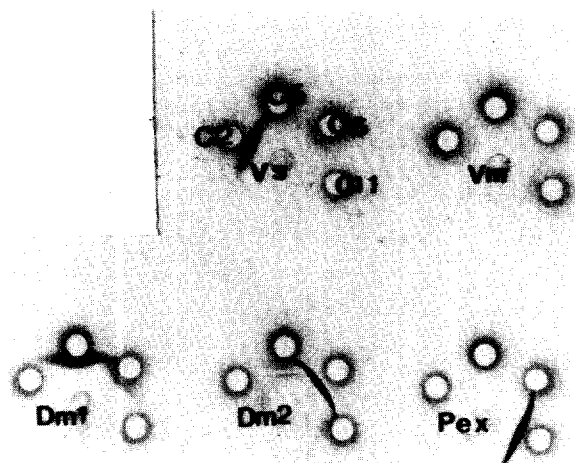


FIG. 4. Gel immunodiffusion patterns of hyperimmune rabbit antisera raised against purified vespid PLs reacting with purified vespid PLs in the central wells. Antiserum C2 was raised against Vs PL, C5 against Dm PL₁, C6 against Dm PL₂, and C11 against Pex PL. The center wells contain 0.5 mg/ml of Vs PL and 1 mg/ml of each of the other PLs.

with Vv but not with Vm, *Vespa*, or *Polistes*. The two antisera against the white-faced hornet isozymes demonstrated different specificities. The anti-PL₁ cross-reacted strongly with all but Vm, and the anti-PL₂ cross-reacted strongly with Vm but only weakly with *Vespa*. It did not react with *Polistes* PL. The antiserum raised against *Polistes* PL was the most specific, reacting strongly with *Polistes*, weakly with Vs, and a possible trace reaction with Vm PL. It is apparent from examining Fig. 5 that the reactivities are not reciprocal between antigen and antisera, suggesting that there are varying dominant determinants.

The results of RAST-inhibition experiments with the purified vespid PLs and 10 vespid-reactive human sera are presented in Table II. All the sera tested were RAST positive to all the venoms used and were known to react strongly with the PL coupled to the RAST disk. Serum 7994 demonstrates cross-reactivity with Vm and Vs but not with *Polistes*. Serum 6642 demonstrates cross-reactivity between *Polistes* and *Vespa*. Serum 6750 exhibits cross-reactivity between *Polistes* and Vm but not Vs. Sera 8568, 10283, and 13263 all demonstrate cross-reactivity between Dm, Vm, and *Polistes* PLs. Serum 4092 exhibits Vs specificity, serum 10873 demonstrates Vm specificity, and serum 10115 demonstrates *Polistes* specificity. Serum 8686 demonstrates cross-reactivity between Vm, Vs, and *Polistes*. As with the experiments with rabbit antisera, the human RAST inhibition studies demonstrate a variety of cross-reactivity patterns, again suggesting that the dominant immunologic determinants vary from one individual to another.

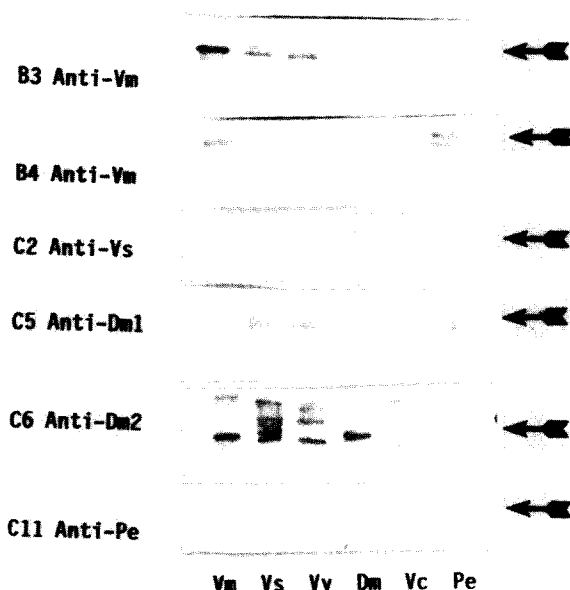


FIG. 5. Immunoblots of SDS-PAGE of vespid venoms reacted with rabbit antisera against vespid PLs and developed with antirabbit IgG horseradish peroxidase. The antisera are labeled on the left. The arrows on the right indicate the approximate position of the major PL band of Vms. The bands of lower molecular weight visible are some of the natural fragments of PL released by reduction.^{1,3,5} There are also some trace reactivities with larger molecules. Antibody C6 appears to have some reactivity with hyaluronidase; 0.5 µg of venom protein was used for each electrophoresis.

DISCUSSION

Peptide mapping studies of PLs isolated from six species of vespids with trypsin, chymotrypsin, staphylococcal V8 protease, and CNBr cleavage indicate that, although the PLs are of similar molecular weight and enzyme specificity, there are significant differences in their structures. This impression is confirmed by examination of the amino acid analyses for some of the PLs in Table I^{2,3,4,9} in which significant differences are evident. Most striking are the differences in number and location of MET and cysteine residues. These residues are usually highly conserved in related sequences. The differences in number of MET residues are reflected in the CNBr maps in Fig. 3. All three enzymes used for cleavage have different specificities. Trypsin cleaves at the carboxyl side of arginine and lysine; chymotrypsin cleaves at the carboxyl side of aromatic residues, and V8 protease cleaves at the carboxyl side of glutamic acid. Three of the four methods of cleavage demonstrated significant differences in primary structure.

Cross-reactivity studies with rabbit antisera demonstrated varying amounts of cross-reactivity, de-

TABLE II. RAST inhibition of purified vespid phospholipases by purified vespid phospholipases

Serum	Specificity	Disk	Inhibitor	Concentration (μ g) for 50% inhibition	% Inhibition by 5 μ g
7994	<i>Vespula</i>	Vs	Vm	7.0	43
			Pex	—	0
			Vs	2.8	65
6642	<i>Vespula</i> ; <i>Polistes</i>	Pex	Vm	1.8	86
6750	<i>Polistes</i>	Pex	Vm	1.3	67
			Vs	—	0
			Pex	6.8	44
8568	Dm	Dm	Vm	0.3	78
			Vs	—	0
			Pex	1.4	80
10283	Dm	Dm	Vm	22	26
			Pex	37	29
13623	Dm	Dm	Vm	1.5	88
			Pex	0.5	92
4092	Vs	Vs	Vm	—	22
10873	<i>Vespula</i>	Vm	Vs	—	2
			Pex	—	4
			Vm	4	53
10115	<i>Polistes</i>	Pex	Vm	—	0
			Vs	—	0
			Pex	6.6	45
8686	<i>Vespula</i> (Vm)	Vs	Vm	0.2	89
			Pex	0.9	81

Sera were selected to illustrate a variety of specificities.

pending on the serum and method used for testing. The most striking finding was the nonreciprocity of the cross-reactions between antigen and antisera. This has been reported with the use of mouse antisera by King et al.⁹ Two of the six antisera were relatively specific, and four were generally cross-reactive.

RAST-inhibition studies with 10 individual human sera elicited similar findings to the rabbit antisera. These sera were all from untreated patients with recent systemic reactions who reacted to multiple vespid venoms. In some cases (e.g., 10873 and 10115) the IgE antibodies were highly specific for a single PL, but in other cases (e.g., 6642, 6750, and 8686) the IgE antibodies demonstrated cross-reactions between genera of vespids. The existence of variable degrees of cross-reactivity among and within genera to homologous proteins is not surprising, since similar observations have been made from RAST-inhibition studies with whole venoms.^{12, 17, 18}

Combining the results of the structural and immunologic studies, it is apparent that there is significant evolutionary divergence among the vespid PLs, even within the genus *Vespula*. Although the molecules are similar in size and general enzyme specific-

ity, they differ significantly in structure, including the number of disulfide loops. There is also some evidence that they differ functionally.⁹ Each also has its own activity modifying peptide found in the venom.²⁰

Studies on the structure and cross-reactivity of vespid allergens have not served to simplify the dilemma of the clinician dealing with the patient who is multiply reactive to vespid venoms. There does not appear to be any reliable method to easily determine the primary insect or allergen to which the patient was sensitized. Some individuals produce antibody that is highly specific for the immunizing PL, but many others produce antibody of varying degrees of cross-reactivity. With the exception of *Polistes*, the hyaluronidases appear to be more cross-reactive⁸ than the PLs, and cross-reactions are even observed between *Vespula* and honeybee hyaluronidases.⁵ The antigen 5 molecules from the various vespids appear to be more similar to each other than the PLs are, but significant heterogeneity of reactivity is also observed with them.^{1, 3-9}

According to the manufacturer, Vespa Laboratories, Division of Pharmacia Inc., Spring Mills, Pa., both yellow jacket- and paper wasp-venom protein ex-

tracts are prepared with standard ratios of individual species. All the important North American species are contained in each batch of extract. For the present it appears that the best course of action for the clinician is to treat with all of the venoms to which the patient is believed to be significantly reactive.

The live insects were collected by Mr. Robert Jacobson. Venom was obtained by Marvin Alligood, Mark Grossnickle, and Robert Jacobson. Charlene Cone and Elizabeth Kopelman assisted with the performance of many of the experiments.

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