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Allergens in Hymenoptera Venom

XXVI: The Complete Amino Acid Sequences of Two Vespid Venom Phospholipases

Key Words

Cross-reactivity
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Venom, hymenoptera
Vespid, yellow jacket

Abstract

The 2 major allergenic proteins in vespid venoms are antigen 5s and phospholipases (PLs). Vespid PLs have a molecular weight of about 34,000 and have been previously shown to have an A₁B specificity, unlike the A₂ specificity of bee PLs. The complete amino acid sequences of the venom PL from the yellow jacket, *Vespula maculifrons*, and the more acidic isoenzyme from the white faced hornet, *Dolichovespula maculata*, have been determined by sequencing overlapping peptides isolated from enzyme and chemical digests of the proteins. *Ves m 1* is composed of 300 amino acids, with variants found at 3 positions. *Dol m 1.02* is composed of 303 amino acids with variants at 2 positions. Comparison with the sequence of *Dol m 1.01* determined by cDNA sequencing gave 66.7% identity with *Dol m 1.01* with almost all variation in the first 131 positions. *Ves m 1* showed 69% identity with *Dol m 1.01* and 38.7% with *Dol m 1.02*. Comparison of the PL sequences with the Protein Identification Resource data base showed many similarities with the lipase family, but very little relationship to known PLs. The amount of structural similarity between *Dol m 1* and *Ves m 1* is similar to that found among antigen 5 molecules of different genera, and is sufficient to account for antigenic cross-reactivity. There appear to be a number of highly conserved regions of the PL molecules, especially in the C-terminal 168 residues. These regions may form common epitopes found in several species and genera of vespids. Antibodies against these common epitopes will not be able to distinguish among PLs from various vespids. The finding of 2 significantly different sequences for *Dol m 1* is parallel to the finding for *Dol m 5*.

Introduction

Phospholipase (PL) A₁B is one of the 2 major allergens of the venoms of vespid wasps [1–6]. The molecules are of about 34,000 molecular weight and usually have an acid-sensitive cleavage point somewhere around the middle. The vespid PLs cleave phosphatidylcholine at the 1 position fatty acid, then at the 2 position, unlike bee and snake venom PLs which cleave only at the 2 position. The vespid PLs are about twice the size of bee PL and do not demonstrate any

significant immunological cross-reactivity with the bee venom enzyme. Since many allergic individuals exhibit reactivity to multiple vespid venoms [7–9], it is probable that some of this multiple reactivity is due to immunologic cross-reactivity of the major allergens. This multiple and cross-reactivity has been demonstrated among individual species of the same genus [10–13] and among different genera of vespids [13, 14]. In a previous paper we have already explored the sequence relationships among antigen 5 molecules from various species and genera of vespid wasps [15].

In this paper we present the complete amino acid sequences of 2 additional vespid venom PLs, from the yellow jacket, *Vespula maculifrons*, and one of the two isoenzymes from the white faced hornet, *Dolichovespula maculata*. In the systematic nomenclature for allergens these are named *Ves m* 1 and *Dol m* 1.02. These 2 protein sequences are compared to the cDNA-derived sequence of *Dol m* 1.01, reported by Soldatova et al. [16] and to all of the protein sequences in the Protein Identification Resource (PIR) database.

Materials and Methods

Venom and PLs

Venom from *V. maculifrons* workers and *D. maculata* workers was obtained by squeezing dissected venom sacs from insects killed by freezing [17]. The venom was fractionated on a Sephadex G75 superfine column, and the PL-containing fractions further purified by high performance cation exchange chromatography, and an additional Sephadex G75 gel filtration [17]. N-terminal amino acid sequencing showed purities of greater than 98% for both PLs. The initial sequencing yields varied from 25 to 35%, and no N-terminal sequences were observed from either antigen 5 or hyaluronidase in either PL preparation. The N-terminal sequence showed no contamination of the acidic phospholipase isoenzyme of *D. maculata* by the basic isoenzyme.

Reduction and Alkylation

Purified proteins were reduced in either 6 M guanidine HCl, 0.5 M Tris-HCl (pH 7.4), 0.01 M EDTA; or 8 M urea in 0.1 M ammonium bicarbonate, (pH 7.8) with 5 mg of dithiothreitol under nitrogen for 2 h at 56°C. The reduced proteins were alkylated with 13.4 mg of iodoacetic acid (Pierce Chemical Co., Rockford, IL, USA) freshly dissolved in 0.1 ml of 0.5 N NaOH, in the dark under nitrogen at room temperature for 1 h [18]. Denaturing agents and excess reactants were removed by dialysis.

Protein Fragmentation

Cyanogen bromide cleavage was performed in 70% formic acid with at least 100-fold excess of CNBr, in the dark under nitrogen at room temperature for 24 h [18]. Ten volumes of water were added and the mixture dried in a centrifugal vacuum evaporator. Sequencing grade staphylococcal V8 protease, trypsin and endoproteinase-ASP-N were all purchased from Sigma Chemical Company, St. Louis, Mo., USA. Digestions were performed in 0.1 M ammonium bicarbonate (pH 7.8) or Tris-HCl (pH 7.4) with up to 2 M urea added if necessary for solubility, with 2–4 µg enzyme/mg protein at 37°C [18, 19]. Enzyme hydrolyses were stopped by adding trifluoroacetic acid (TFA; Pierce Chemical Co.).

Separation of Peptides

Peptide digests were separated by high performance chromatography, using an 0.46×25 cm Vydac 218TP54 reverse phase column (The Separations Group, Hesperia, Calif., USA) with a gradient starting at 0.1% TFA in water to 70% of 0.07% TFA in acetonitrile. The flow rate was 0.6 ml/min and 0.5 min fractions were collected in microcentrifuge tubes. The separation was monitored at 220 nm.

Protein Sequencing

Sequencing was performed on a Milligen-Bioscience Model 6625 Prosequencer (Millipore Corp., Marlborough, Mass., USA) This is a solid phase fully automated sequencer/PTH analyzer. Proteins were coupled to either DITC disks or arylamine disks, and the covalently bound protein was reduced and alkylated in the presence of 6 M guanidine HCl as described above. The disks were then washed 5 times each in water and methanol and air dried. Separated peptides from digests were coupled to arylamine disks for sequencing. In rare cases peptides were coupled to DITC disks to verify aspartic or glutamic acid residues.

Alignment and Database Comparisons

The Hitachi Software Engineering America, Ltd. (Brisbane, Calif., USA) Hibio Prosis programs and compact disk edition of the PIR (National Biomedical Research Foundation) and SWISS-PROT (European Molecular Biology Laboratory) databases were used on an MS-DOS computer system. The program can automatically align pairs of sequences, and a manual alignment routine is provided for multiple sequences. The PIR was release 29 and SWISS-PROT release 20.

Results

The sequence of *Ves m* PL was determined from an N-terminal sequence and by sequencing all of the peptides isolated from staphylococcal V8 protease, trypsin and cyanogen bromide digests. The final sequence and the overlapping peptides used to establish it are shown in figure 1. The dilute acid cleavage seen in SDS-PAGE [2] was found to occur between residues 165 and 166, aspartic acid and proline. Variants were found at 3 positions in the sequence, methionine for alanine at position 97, proline for isoleucine at 191 and leucine for isoleucine at 202. At position 191 the ratio of I to P was 1.50 and at position 202 the ratio of I to L was 1.47. The methionine variant at position 97 was found by the generation of a CNBr fragment, and was not quantifiable. The sequence contained 300 amino acids, giving a molecular weight of 33,539. There were 13 cysteine residues as were found in the cDNA sequence for *Dol m* 1.01 [16]. Despite the odd number of cysteines, the molecules exist primarily as monomers, suggesting that there is a free sulfhydryl group. Only a single tryptophan residue was found at position 82, also similar to *Dol m* 1.01. Computer comparison of the sequences of *Ves m* 1 and *Dol m* 1.01 as shown in figure 2 showed 68.3% identity with only 15 of 95 differences requiring more than a single base change. The C-terminal 173 residues showed a very high degree of similarity. No insertions or deletions were required to align all 13 cysteine residues. Comparison of the sequence of *Ves m* 1 with the partial cDNA sequence of *Ves v* 1 [20] showed 95.3% identity over the first 258 residues, with only 3 non-conservative changes.

Fig. 1. The complete amino acid sequence of the yellow jacket venom PL molecule, *Ves m 1*. The sequence was determined from overlapping peptides indicated by the symbols below the single letter amino acid codes.

— = N-terminal sequence; — = staphylococcal V8 protease peptides; ***** = tryptic peptides; — = cyanogen bromide peptides. The vertical lines indicate junctions between peptides. Alternative residues were found at 3 positions: M for A at position 97; P for I at position 191, and L for I at position 202.

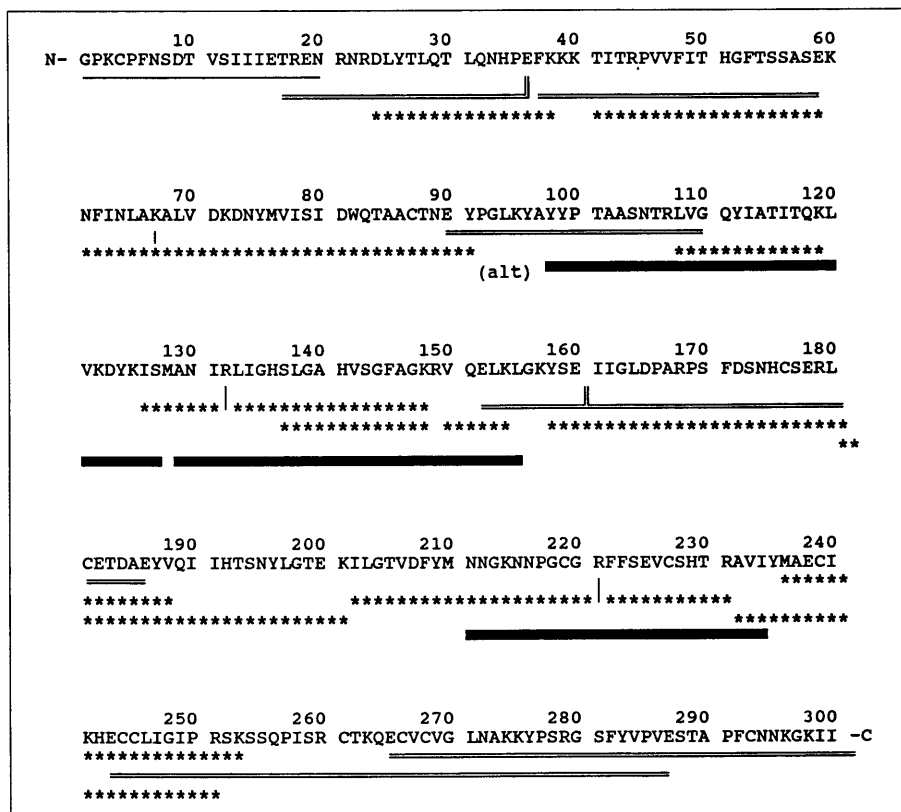


Fig. 2. Comparison of the protein sequence of *Ves m 1* with the translated cDNA sequence of *Dol m 1.01* [16]. Double dots between the residues indicate identity, and single dots conservative amino acid substitutions due to single base changes. The molecules show 68.3% identity.

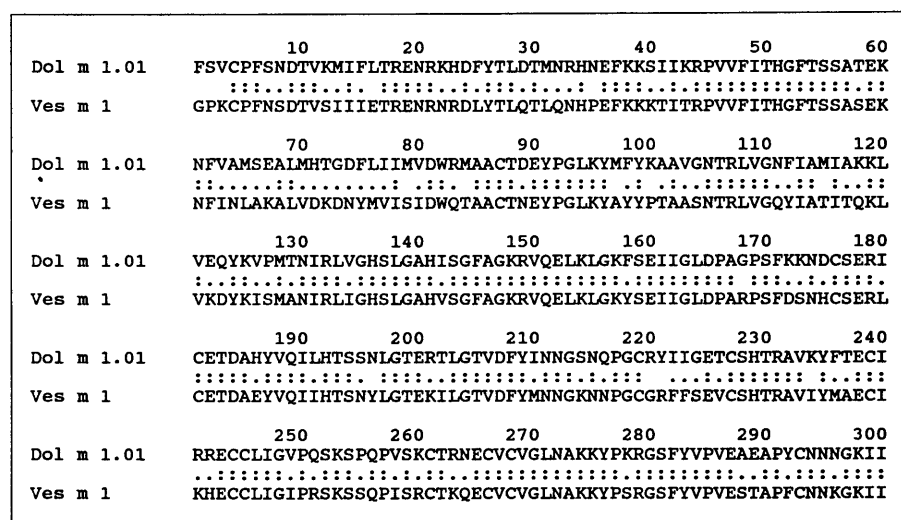


Fig. 3. The complete amino acid sequence of the acidic PL isoenzyme of white faced hornet venom, *Dol m* 1.02, as determined by sequencing overlapping peptides. The symbols underneath the amino acid codes indicate the peptides used to establish the structure. — = N-terminal sequence; ■ = cyanogen bromide peptides; **** = tryptic peptides; === = staphylococcal V8 protease peptides; 1111 = endoproteinase-aspartic acid-N peptides; aaaa = dilute acid hydrolysis. Alternative residues were found at 2 positions: E for G position 55, and Y for F at position 295.

The complete amino acid sequence of *Dol m* 1.02 was determined by sequencing the N terminus and all of the peptides isolated in pure form and sufficient yield from staphylococcal V8 protease, trypsin, endoproteinase-ASP-N, cyanogen bromide and dilute TFA digestions as is shown in figure 3. The sequence consisted of 303 amino acid residues giving a molecular weight of 33,780. There were 13 cysteines and a single tryptophan. Two additional residues were inserted at the N terminus. Two variant positions were found, glutamic acid for glycine at position 55 and tyrosine for phenylalanine at position 295. At position 295 the ratio of tyrosine to phenylalanine was 5.6. Glycine was the predominant residue at position 55, glutamic acid could not be reliably quantitated because of the sequencer chemistry used, but its presence was confirmed by the isolation of a staphylococcal V8 fragment beginning at position 56. The acid cleavage point was found between residues 168 and 169, D and P. The comparison of the protein sequence of *Dol m* 1.02 with the translated cDNA sequence of *Dol m* 1.01 [16] is shown in figure 4. There is 66.7% identity over the 303 amino acid residues. The regions from residue 131 of *Dol m* 1.02 and 130 of *Dol m* 1.01 are highly conserved. But there

The comparison of the sequences of *Dol m* 1.01, 1.02 and *Ves m* 1 is shown in figure 5. The sequences of *Dol m* 1.02 and *Ves m* 1 show 58.7% identity with only a few short conserved areas in the N-terminal 130 residues. Much of the C-terminal regions of all 3 proteins are similar with only a number of short regions showing differences.

Fig. 4. Comparison of the amino acid sequences of the 2 isoenzymes of white faced hornet venom PL, *Dol m* 1.01 and 1.02. A dash in the sequence represents a position inserted to maximize homology. Double dots between the sequences indicate identical residues and single dots residues that differ by a single base change. The molecules show 66.7% identity.

		10	20	30	40	50
<i>Dol m</i> 1.01		FSVCPFSNDTVKMIFLTR	ENRKHDFYTL	DTMNRHNEFKS	-IIKRPVVF	ITHGFTSSATE
<i>Dol m</i> 1.02		GILPECKLVPEEISFVLSTREN	RDGVYLT	LQKLKNGKMF	KNSDLSSKKVP	FLIHGFISSATN
		10	20	30	40	50
		60	70	80	90	100
<i>Dol m</i> 1.01		KNFVAMSEALMHTGDFLI	IMVDWRMAACT	DEYPLKYM	FYKAAVGNTRL	VGNFIAMI
<i>Dol m</i> 1.02		KNYADMTRALLDKDDIM	VISIDWRDGAC	SNEFALLK	FIGYPKAVENTRA	VGYIADFSKI
		70	80	90	100	110
		120	130	140	150	160
<i>Dol m</i> 1.01		LVEQYKVPMTNIRLVGHS	LAGAHISGFAG	KRVQELKLG	FSEIIGLDPAG	PSFKKND
<i>Dol m</i> 1.02		LIQYKVLLENIRLVGHS	LAGAIAAGFAG	KEFQRFKLG	YPEIIGLDPAG	PSFKKDCPER
		130	140	150	160	170
		180	190	200	210	220
<i>Dol m</i> 1.01		ICETDAHYVQILHTSS	NLGTERTLGT	VDFYINNGSN	QPGCRYIIGET	CSHTRAVKYFTEC
<i>Dol m</i> 1.02		ICETDAHYVQILHTSS	NLGTERTLGT	VDFYINDGSN	QPGCTYIIGET	CSHTRAVKYLTEC
		190	200	210	220	230
		240	250	260	270	280
<i>Dol m</i> 1.01		IRRECCLI	GVPSKSPQ	PSKCTRNE	CVGVLNAK	KYPKRG
<i>Dol m</i> 1.02		IRRECCLI	GVPSKSPQ	PSKCTRNE	CVGVLNAK	YKRG
		250	260	270	280	290
		290	300			

Fig. 5. Comparison of the amino acid sequences of *Dol m* 1.01, *Dol m* 1.02 and *Ves m* 1. * = Residues identical to those in *Dol m* 1.01, - = positions added to maximize homology. The sequences of *Dol m* 1.02 and *Ves m* 1 show 58.7% identity.

		10	20	30	40	50
<i>Dol m</i> 1.01	1	--FSVCPFSN	DTVKMIFLTR	ENRKHDFYTL	DTMNRHNEFK	KS-IIKRPVV
<i>Dol m</i> 1.02	1	GILPE*KLVP	EEISFVLS**	***DGVYL**	QKLKNGKM**	N*DLSSKK*P
<i>Ves m</i> 1	1	--GPK***NS	***SI*IE**	***NR*L**	Q*LQN*P**	*K-T*T***
		60	70	80	90	100
<i>Dol m</i> 1.01	51	FITHGFTSSA	TEKNFVAMSE	ALMHTGDFLI	IMVDWRMAAC	TDEYPLKYM
<i>Dol m</i> 1.02	51	*LI***I***	*N**YAD*TR	**LDKD*IMV	*SI***DG**	SN*FAL**FI
<i>Ves m</i> 1	51	*****NS	S****INLAK	**VDKDNMYV	*SI**QT***	*N*****A
		110	120	130	140	150
<i>Dol m</i> 1.01	101	FYKAAVGNTR	LVGNFIAMIA	KKLVEQYKVP	MTNIRLVGHS	LAGAHISGFAG
<i>Dol m</i> 1.02	101	G*PK**E***	A**KY**DFS	*I*IQK***L	LE*****I***	***Q*A****
<i>Ves m</i> 1	101	Y*PT*AS***	***QY**T*T	Q***KD**IS	*A****I***	***V*****
		160	170	180	190	200
<i>Dol m</i> 1.01	151	KRVQELKLGK	FSEIIGLDP	AGPSFKKND	CSERICETDAHY	VQILHTSSNL
<i>Dol m</i> 1.02	151	*EF*RF***	YP*****	*****K*P	*****L*	*****N*
<i>Ves m</i> 1	151	*****NS	Y*****S	R***DS*H**	**L*****E*	***I***NY*
		210	220	230	240	250
<i>Dol m</i> 1.01	201	GTERTLGTVD	FYINNGSNQP	GCRYIIGETC	SHTRAVKYFT	ECIRRECCLI
<i>Dol m</i> 1.02	201	*****NS	***D*****	**T*****	*****L*	*****N*
<i>Ves m</i> 1	201	***KI*****	**M***K*N*	**GRFFS*V*	*****I*MA	***KH*****
		260	270	280	290	300
<i>Dol m</i> 1.01	251	GVPQSKSPQP	VSKCTRNECV	CVGLNAKYP	KRGSFYVPE	AEAPYCNNG KII
<i>Dol m</i> 1.02	251	*****N***	*****K***	*****E***	*K*****	*K**F*****
<i>Ves m</i> 1	251	*I*R***S**	I*R**KQ***	*****S*	S*****	ST**F***K* ***

The closest relationships were to vertebrate lipoprotein lipases (triacylglycerol lipases). These enzymes aligned with the region from residues 99 to 220 of the PLs; probably indicating the lipid-binding regions. A lesser degree of matching was seen from residues 15 to 98. Protein groups also found to match include insect vitellogenins and other insect yolk proteins, multidrug resistance proteins, electron transport proteins and numerous hemoglobin β chains. *Dol m* 1.02 showed matches with Indian elephant hemoglobin β chain from residues 99 to 166 with maximum similarity

from residues 116 to 153. In addition a number of shorter regions of sequence were found that matched with calcium-binding proteins as well as with other cation-binding proteins, and also with some kinases; but matches were not consistently found in the same regions of the venom PLs. No clear relationship was seen with PL A₂ molecules, the first match found with a PL A₂ molecule was the 577th highest score, with a match score significantly lower than that for hemoglobin β chains.

Discussion

The complete protein sequences of 2 vespid venom PLs have been determined. The sequence of *Ves m 1* is very similar to that reported for *Ves v 1*, determined from cDNA [16]. The degree of similarity is comparable to that seen among the antigen 5s from the vulgaris species group [15]. The sequence of *Dol m 1.02* is only 66.7% identical with the reported cDNA sequence of *Dol m 1.01* [16], and also contains 3 additional residues. The differences are generally not conservative and are concentrated in the N-terminal half of the molecule. This is also consistent with the findings for *Dol m 5*, which has 2 isoallergens differing by 23% and also containing different numbers of residues [22]. The N-terminal regions of *Dol m 1.01* and *1.02* have so little similarity that nucleic acid probes designed for one would not hybridize with the other. Of all the vespid venoms that have been studied only that from *Dol m* has pairs of both proteins exhibiting major structural differences. Minor differences are seen in antigen 5s and PLs from other vespid species, but these are usually conservative changes involving only 1–3% of the residues [15]. The 2 isoallergens of *Dol m 1* were found in every batch prepared in our laboratory, including insects collected locally and from other regions of the country. Batches prepared from a single nest also contained both forms of PL as has been found for *Dol m 5* [15]. We have been unable to find any studies of the genetics of *Dol m* which might shed light on the reason for these findings.

The vespid venom PLs are about twice the molecular weight of bee venom PLs and the amino acid sequences show no significant similarity [22, 23]. The vespid PLs appear to be derived from the lipase family of proteins, rather than the PL A₂ family. The vespid PL are 31.2% identical over 215 amino acids with mammalian pancreatic triacylglycerol lipases. Immunologic cross-reactivity is not found between bee and vespid PLs with either human or animal antibodies. Comparisons with the PIR protein structure database also suggest regions that may be involved in calcium ion binding. The relationship to hemoglobin β chains is interesting, but of unknown significance. Homologies with hemoglobin chains raised from 19 to 31% identity over ranges of 55–69 amino acids.

The sequence data reported here and from cDNA sequencing [16] help to explain and clarify the immunologic [4] and biochemical [6] findings reported previously. The differing amino acid compositions between the 2 forms of *Dol m 1* and between *Dol m 1s* and *Ves m 1* are consistent with the amount of sequence variance. In all 4 vespid PLs studied the 13 cysteines are conserved. The number of methionines is variable with *Dol m 1.01* having 9 and *Dol m 1.02*

having 3. This was illustrated in the peptide map in figure 3 of Hoffman [6], where many more fragments can be seen for the basic isoenzyme (called Dm2 by Hoffman), *Dol m 1.01*. The differences in the proteolytic enzyme-generated maps are also consistent with the sequence differences found. The immunodiffusion studies with rabbit antisera [6] show antiserum C5 which precipitates strongly with only *Dol m 1.02*, but which does cross-react by immunoblot with some other vespid phospholipases. Antiserum C6 precipitates with both isoenzymes from *Dol m*, and shows a different pattern of cross-reactivity in immunoblots. Antiserum B3 raised against *Ves m 1* reacts only with yellow jacket PLs, while antiserum B4 also raised against *Ves m 1* is cross-reactive against hornet and wasp PLs. RAST and RAST inhibition studies [4, 6] also demonstrate the presence of both specific and cross-reactive populations of IgE antibodies in different patient sera. The cross-reactive antibodies probably result from responses to the conserved regions of the molecules, and the specific antibodies from responses to the more variable regions. The differences between *Dol m 1.01* and *1.02* are certainly large enough to generate antibodies that recognize one or the other isoallergen, as has been shown with rabbit antisera [6]; but not yet been demonstrated in man. Berzofsky and Schechter [24] have classified cross-reactivity into 2 types: shared cross-reactivity which results when 2 proteins contain an identical epitope, and true cross-reactivity that results from an epitope that is different from but structurally related to the epitope in the immunizing antigen. Both types of cross-reactivity may be present in antibodies to vespid venom PLs, since they contain sequence regions that could generate both identical and conformationally similar 3-dimensional structures. Antibodies that exhibit shared cross-reactivity will have equal affinities for both antigens, since the epitopes are identical.

Information from the amino acid sequences of antigens can be used to find T-cell epitopes and some B-cell epitopes. Human IgE antibodies against *Ves m 1* appear to react primarily with epitopes of the native conformation [25]. Completely reduced and alkylated *Ves m 1* has an inhibition potency of about 10^{-5} relative to native PL. Many allergic sera reactive with native *Ves m 1* do not recognize the denatured *Ves m 1* in SDS-PAGE immunoblots [25]. An extensive discussion reviewing present knowledge of the structural basis of antigenic specificity and design of synthetic vaccines has recently been published [26].

The sequences of the 4 vespid PL molecules currently known show that many of the yellow jackets have very similar antigens, which would be expected to be almost totally cross-reactive. White faced hornets have 2 types of PL molecules which are 33% different from each other with the

first half of the molecules having only a few common features. The hornet and yellow jacket enzymes have only 59–69% identity. As is well known, many venom-allergic patients have IgE antibodies that cross-react among the vespid venoms, many others produce specific IgE antibodies. It is improbable that a single natural or synthetic PL would be sufficient for the reliable diagnosis and immunotherapy of the PL component of vespid venom allergy, since allergic patients have IgE antibodies to both common and specific determinants. The studies reported here help to clarify the structural basis for immunologic cross-reactivity, and to ex-

plain the complexity of previously reported clinical and immunological results.

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