Yellow jacket venom allergens, hyaluronidase and phospholipase: Sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy

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Three known allergens of yellow jacket (Vespula vulgaris) *venom are antigen 5, hyaluronidase, and phospholipase. Yellow jacket antigen 5 has been previously cloned and expressed in bacteria; it contains 204 amino acid residues, and it has 69% and 60% sequence identities with the homologous proteins of white-faced hornet* (Dolichovespula maculata) *and wasp* (Polistes annularis), *respectively. These studies are now extended to yellow jacket hyaluronidase and phospholipase; they contain 331 and 300 amino acid residues, respectively, and they show 92% and 67% sequence identity with their homologs of white-faced hornet. Tests with the natural and the recombinant vespid allergens in mice indicate partial antigenic cross-reactivity of their homologous proteins at both B- and T-cell levels. There is greater cross-reactivity among hornet and yellow jacket allergens than that among hornet or yellow jacket and wasp allergens. The order of cross-reaction of the three vespid allergens is hyaluronidase > antigen 5 > phospholipase. The continuous (linear) B-cell epitopes of vespid allergens show greater cross-reactivity than their discontinuous epitopes do. The discontinuous B-cell epitopes are immunodominant for all vespid allergens. The low degree of cross-reactivity of the immunodominant discontinuous B-cell epitopes of vespid allergens should be taken into consideration in selection of venoms for immunotherapy of patients with sensitivity to multiple vespids. (J Allergy Clin Immunol 1996;98:588-600.)*

Key words: *Allergens, yellow jacket, antigen 5, hyaluronidase, phospholipase, hornet, wasp*

Patients allergic to stinging insects often show multiple sensitivity to different vespids, which include hornets, yellow jackets, wasps, and bees. For example, in one survey of several hundred serum samples evaluated by RAST,[1] about 80% of patients allergic to yellow jackets are reported to have hornet sensitivity, about 30% of patients allergic to yellow jackets and/or hornets have wasp sensitivity, and about 45% of patients allergic to bees have vespid sensitivity. Inhibition of RAST analysis showed that there are varying extents of antigenic cross-reactivity of venoms, depending on the patient sera.[2-5] These results reflect in part the variable immune responses of patients to venoms because venoms are mixtures of allergens.

Abbreviations used

PCR:	Polymerase chain reaction
RACE:	Rapid amplification of 3′ or 5′ cDNA ends
SDS:	Sodium dodecylsulfate

There are three known vespid venom allergens: antigen 5 (of unknown biochemical function), hyaluronidase, and phospholipase A_1.[6] The primary structures of several hornet, yellow jacket, and wasp antigen 5s,[7,8] one hornet *(Dolichovespula maculata)* hyaluronidase,[9] and two hornet *(D. maculata* and *Vespa crabo)* and two yellow jacket *(Vespula maculifrons* and *V. squamosa)* phospholipases[10-12] are known. The cloning and expression

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of yellow jacket *(V. vulgaris)* hyaluronidase and phospholipase, Ves v 1 and Ves v 2, respectively, are reported in this article. The recombinant allergens lack the disulfide bonds of the natural allergens, and presumably they differ from natural allergens in their conformations. Nonetheless, they are useful for studies of the linear or continuous type of B-cell and T-cell epitopes.

We have previously studied the antigenic cross-reactivities of individual vespid venom natural allergens by inhibition analysis with specific mouse sera.[13] We have now extended these earlier studies to include cross-reactivities of their continuous and discontinuous B-cell and T-cell epitopes.

METHODS

Isolation of vespid venom allergens

Antigen 5s, hyaluronidases, and phospholipases were isolated from hornet *(D. maculata)*, wasp *(P. annularis)*, and yellow jacket *(V. vulgaris)* venom sac extracts (Vespa Lab, Spring Mills, Pa.), as described previously.[14, 15] Briefly, the phospholipase component was isolated from venom sac extract by affinity chromatography on an adsorbent containing a substrate analog of phospholipase, followed by affinity chromatography on heparin Sepharose (Pharmacia, Uppsala, Sweden) to separate antigen 5 and hyaluronidase.

Reduced vespid phospholipases

The proteins (15 to 30 μmol/L) were reduced with 0.03 mol/L dithiothreitol in 6 mol/L guanidine hydrochloride + 0.2 mol/L Tris-HCl (pH 8.6) at ambient temperature for more than 16 hours. The entire mixture was applied to a 4 × 0.9 cm column of VYDAC protein and peptide C18 silica (The Separation Group, Hesperia, Calif.), which was eluted with a linear 0.5% per milliliter gradient of 2-propanol in 0.1% trifluoroacetic acid at 60 ml/hr. The reduced proteins were eluted at about 40% 2-propanol, and volume was reduced by lyophilization. Then the protein solution of about 15 μmol/L was stored at 4° C until use. Complete removal of solvent was avoided because it is difficult to resolubilize the dried reduced protein.

Partial amino acid sequences of yellow jacket phospholipase Ves v 1 and hyaluronidase Ves v 2

Partial amino acid sequences were determined by Edman degradation of the intact protein or its fragments on digestion of the reduced and S-carboxymethylated protein with endoproteinase Glu-C (Pierce Chemical Co., Rockford, Ill.), according to the procedures described in our previous publication.[16]

Complementary DNAs of Ves v 1 and 2

Total RNAs were isolated from venom acid glands of *V. vulgaris*.[16] Ves v 1– and Ves v 2–specific cDNAs were prepared from total RNAs according to Frohman's procedure[17, 18] for rapid amplification of 3′ or 5′ cDNA ends (RACE). For Ves v 2 cDNA, a modified form of 3′ RACE[19] with a single target-specific sense primer was used.

First-strand cDNAs for 3′ RACE were synthesized from the MeHgOH-denatured total RNAs (5 μg) as the template, with oligonucleotide number 1 (Table I) as the primer, by using the cDNA synthesis system from Gibco-BRL (Bethesda, Md.). For 5′ RACE of Ves v 1 cDNA, the single-stranded cDNAs were synthesized as described previously, except that oligonucleotide number 5 (Table I) was used as the primer, then a poly-dA tail was formed with terminal deoxynucleotidyl transferase (U.S. Biochemical, Cleveland, Ohio). RACE (3′ or 5′) was carried out with GenAmp polymerase chain reaction (PCR) reagent kit (Perkin-Elmers Cetus, Norwalk, Conn.) with Amplitaq polymerase. PCR was carried out for two successive rounds of 30 cycles as follows: template, 2 μmol/L each sense and anti-sense primers, 400 μmol/L each deoxyribonucleoside triphosphates, and 5 units Taq polymerase (Perkin Elmer Cetus) in 50 μl of 10 mmol/L Tris-HCl (pH 8.4) + 50 mmol/L KCl + 1.5 mol/L $MgCl_2$. Each cycle consisted of 45 seconds at 94° C, 25 seconds at 50° C, and 3 minutes at 72° C. The amount of template for the first round of PCR was equivalent to 50 ng of total RNAs used for cDNA synthesis, and that for the second round of PCR was 1/1000 of the first PCR mixture.

PCR products were examined by electrophoresis in agarose gel with ethidium bromide staining and by Southern blot analysis as necessary. PCR products, which contain single 3′-overhanging A-nucleotides,[20] were used for cloning into the pCR vector with compatible T-nucleotide overhangs (Invitrogen Corp., San Diego, Calif.). Plasmid DNAs were isolated by using the Wizard Miniprep system (Promega, Madison, Wis.). DNA sequences were determined by the dideoxynucleotide chain-termination method with alkaline-denatured plasmid DNAs and the Sequenase Version 2.0 kit (U.S. Biochemical). Alternatively, they were determined by the automated fluorescence technique on a Perkin Elmer/Applied Biosystems Model 373 Sequencer (Perkin Elmer Cetus).

Expression of vespid allergens

The necessary cDNAs were obtained by PCR amplification of venom cDNAs or modified pCR plasmid template containing the desired cDNA. The necessary primers are listed in Table I. The sense and anti-sense primers were designed to contain, respectively *Bam*HI and *Bgl* II or *Hind*III restriction sites at their 5′ ends.

After phenol-chloroform extraction and ethanol precipitation, the amplified cDNA (about 2 μg) was digested in 60 μl of universal buffer with 20 units each of restriction enzymes *Bam*HI and *Bgl* II or *Hind*III (Gibco-BRL) at 37° C overnight. The product was isolated by electrophoresis in 1.4% LGT agarose (FMC,

TABLE I. Oligonucleotide primers for cloning and/or expression of yellow jacket and hornet phospholipases, Ves v 1 and Dol m 1, and yellow jacket hyaluronidase, Ves v 2

No.	Primer	Notes
1.	AAG GAT CCG TCG ACA TCG ATA ATA CGA CTC ACT ATA GGG ATT T_{15}	cDNA synthesis of 3′ RACE or first-round 5′ RACE
2.	AAG GAT CCG TCG ACA TC	First-round 3′ RACE or second-round 5′ RACE
3.	GAC ATC GAT AAT ACG AC	Second-round 3′ RACE
	F^{1} P K C P F N^{7}	
4.	TTY CCI AAR TGY CCI TTY AA	PCR of residue 1-245 of Ves v 1
	C^{245} C E H K I C E^{238}	
5.	CA RCA YTC RTG YTT IAT RCA YTC	PCR of residue 1-245 of Ves v 1 and cDNA synthesis of 5′ RACE
	T^{54} S S A S E^{59}	
6.	ACT TCA TCT GCA AGT GA	First-round 3′ RACE of Ves v 1
	H^{136} S L G A H^{141}	
7.	CAT AGC TTA GGA GCA CA	Second-round 3′ RACE of Ves v 1
	A^{140} G L S H G^{135}	
8.	GC TCC TAA GCT ATG TCC	First-round 5′ RACE of Ves v 1
	E^{59} S A S S T^{54}	
9.	TC ACT TGC AGA TGA AGT	Second-round 5′ RACE of Ves v 1
	G^{1} P K C P F^{6}	
10.	CGT GGA TCC GGA CCC AAA TGT CCT TTT	Expression of Ves v 1 in pQE12
	I^{300} I K G K N^{295}	
11.	CGT AGA TCT AAT TAT CTT CCC CTT GTT	Expression of Ves v 1 in pQE12
	F^{1} S V C P F^{6}	
12.	CGT GGA TCC TTC TCC GTA TGT CCC TTT	Expression of Dol m 1 in pQE8
	I^{300} I K G N N^{295}	
13.	CGT AAG CTT AAT TAT TTT CCC GTT GTT	Expression of Dol m 1 in pQE8
	A^{102} V G N T R^{107}	
14.	CGT GGA TCC GCC GTT GGT AAT ACA CGC	Expression of Dol m 1 fragment M in pQE12
	Y^{221} R C G P Q^{216}	
15.	CGT AGA TCT ATA TCT GCA ACC GGG TTG	Expression of Dol m 1 fragment M in pQE12
	F^{8} N I Y W N^{13}	
16.	CGT GGA TCC TTC AAY ATI TAY TGG AA	PCR of residues 8-266 of Ves v 2
	Q^{266} Y V Y W W^{261}	
17.	TG RTA IAC RTA CCA CCA	PCR of residues 8-266 of Ves v 2
	S^{1} E R P K R^{6}	
18.	CGT GGA TCC GAG AGA CCG AAA AGA	3′ RACE of Ves v 2 or expression in pQE8
	N^{331} V A E T V^{326}	
19.	CGT AAG CTT GTT GAC GGC TTC TGT CAC	Expression of Ves v 2 in pQE8

Rockland, Maine). The gel slice containing the cDNA of interest was cut out, and 2 to 4 μl of the melted gel (40 to 80 fm cDNA) was used for ligation with 18 ng (8 fm) of appropriately cut and dephosphorylated pQE-12 or -8 vector (QIAGEN, Chatsworth, Calif.). Ligation was carried out overnight at 15° C in the presence of 2 units of T4 DNA ligase in 60 μl of buffer as supplied by the manufacturer (Gibco-BRL).

Competent M15 [pREP4] cells were transformed with the above ligation mix, then grown on laked blood agar plates containing 25 μg/ml kanamycin and 100 μg/ml ampicillin as described by the manufacturer (QIAGEN). Selected colonies were screened by sodium dodecylsulfate (SDS) gel electrophoresis for expression of the expected protein after induction with 1 mmol/L isopropyl β-D-thiogalactoside for 1.5 to 5 hours.

Positive colonies were grown in a culture volume of 250 ml. The collected bacteria pellet was washed free of culture medium with buffer of 0.05 mol/L Tris-HCl (pH 8.0) + 0.10 mol/L NaCl + 1 mmol/L ethylenediaminetetraacetic acid, then dissolved in 6 ml of 6 mol/L guanidine hydrocloride + 0.10 mol/L Tris-HCl (pH 8.0) + 20 mmol/L 2-mercaptoethanol and heated to 100° C for 5 minutes. After clarification by high-speed centrifugation, the solution was applied to a 0.8 × 3.5 cm column of Ni-NTA agarose (QIAGEN). The column was washed in succession with 6 ml portions of 6 mol/L urea + 20 mmol/L 2-mercaptoethanol buffered at pH 8.0, 6.3, 5.9, and 4.5. Recombinant proteins were eluted with 6 mol/L urea buffered with 0.14 mol/L NH_4Ac at pH 4.5.

Recombinant proteins were freed of urea and buffer salts by chromatography on an 8 × 0.9 cm column of VYDAC C18 silica with a linear 2-propanol gradient of 0.5% per milliliter in 0.1% trifluoroacetic acid. The recombinant proteins were eluted at about 40% 2-propanol. After lyophilization, they were found to be soluble in 0.02N HOAc and their concentrations were determined by absorbance at 280 nm. The molar extinction coefficients of proteins at 280 nm were calculated from their tyrosine and tryptophan contents, with ϵ values of 1280 and 5690, respectively. Solutions of recombinant proteins were stored at 4° C. Freezing and thawing of these solutions were avoided because they led to protein precipitation. This was particularly apparent with recombinant hyaluronidases.

Gel electrophoresis and immunoblot

SDS slab gel electrophoresis was carried out in 12.5 or 15.0% polyacrylamide gel.[21] Samples were reduced by boiling in sample buffer + 1% 2-mercaptoethanol for 5 minutes before electrophoresis. For staining with Coomassie Brilliant Blue R-250, 2 to 3 μg of each protein sample was used. For immunoblotting, about 100 or 200 ng of each sample was used. The proteins in the polyacrylamide gel were electroblotted onto nitrocellulose paper in a Model TE 70 Semi-Phor transfer unit (Hoeffer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's directions. The nitrocellulose paper was kept for 0.5 hour in diluent buffer (see Immunization and immunoassays), then in succession for 1-hour periods with 1:100 or 1:300 diluted mouse anti-sera specific for natural or recombinant protein and 1:100 diluted sheep anti-mouse IgG conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.). The blots were stained in a substrate solution of 1.5 mg/ml 4-chloronaphthol and 0.01% H_2O_2 in 0.05 mol/L Tris-HCl (pH 8.0).

Immunization and immunoassays

Groups of four female BALB/c mice, 8 to 10 weeks of age (Jackson Laboratories, Bar Harbor, Maine), were immunized intraperitoneally, each with 0.2 ml of 10 μg/ml immunogen + 5 mg/ml alum in 0.05 mol/L sodium phosphate (pH 6.4) in weeks 0, 2, 4, 6, and 8. Sera were collected by retro-orbital puncture 1 week after each immunization.

Antibodies were measured by enzyme-linked solid-phase immunoassay. Microtiter wells were coated overnight with 5 μg/ml antigen in 0.05 mol/L Tris-HCl (pH 8.0). Any remaining reactive sites of wells were blocked with diluent buffer, which contained 0.1 mg/ml bovine serum albumin, 0.5 mg/ml Tween-20, 0.5 mol/L NaCl, and 0.05 mol/L Tris-HCl (pH 8.0). The wells were then treated for 1-hour periods in succession with varying concentrations of mouse sera to be tested, 10 μg/ml rabbit antibody specific for mouse IgGs, and 1:100 diluted goat antibody specific for rabbit IgG conjugated with horseradish peroxidase. Finally, the bound peroxidase was detected by absorbance at 490 nm after incubation with a substrate solution of 16 mg/ml phenol, 0.5 mg/ml 4-aminoantipyrine, and 0.005% of H_2O_2 in 0.1 mol/L sodium phosphate (pH 7.0). Antibody titers were expressed as reciprocal dilutions required to produce an absorbance change of 1.0 in 30 minutes. Antigen and antibody solutions were prepared in diluent buffer.

Proliferation assays were done with spleen cells from two mice, 10 days after four or five biweekly immunizations. Spleen cells (2×10^5) were cultured with varying concentrations of test antigen in 0.2 ml of culture medium at 37° C in 5% CO_2. Tritiated thymidine (1 μCi) was added on day 3, and the uptake of thymidine was counted on day 4. The culture medium consisted of RPMI-1640 (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffered at pH 7.3 (Sigma Chemical Co.), and 54 μmol/L 2-mercaptoethanol.

RESULTS

Cloning and expression of yellow jacket phospholipase, Ves v 1

Partial amino acid sequence data were obtained for the intact protein and its CNBr cleavage products. Two degenerate primers, 4 and 5 in Table I, were synthesized on the basis of amino acid sequence data and were used in the PCR to amplify a fragment of Ves v 1–specific cDNA from total venom cDNAs. The sequence data of this fragment, which was later shown to encode residue 1-245 of Ves v 1, allowed the synthesis of nondegenerate primers 6 to 9 in Table I. These four primers, 6 to 9, were used in 3′ and 5′ RACEs to

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Ves m   GPKCPFNSDT VSIIIETREN RNRDLYTLQT LQNHPEFKKK TITRPVVFIT    50
Ves v   GPKCPFNSDT VSIIIETREN RNRDLYTLQT LQNHPEFKKK TITRPVVFIT    50
Dol m   FSVCPFSNDT VKMIFLTREN RKHDFYTLDT MNRHNEFKKS IIKRPVVFIT    50

Ves m   HGFTSSASEK NFINLAKALV DKDNYMVISI DWQTAACTNE YPGLKYAYYP   100
Ves v   HGFTSSASET NFINLAKALV DKDNYMVISI DWQTAACTNE AAGLKYLYYP   100
Dol m   HGFTSSATEK NFVAMSEALM HTGDFLIIMV DWRMAACTDE YPGLKYMFYK   100

Ves m   TAASNTRLVG QYIATITQKL VKDYKISMAN IRLIGHSLGA HVSGFAGKRV   150
Ves v   TAARNTRLVG QYIATITQKL VKHYKISMAN IRLIGHSLGA HASGFAGKKV   150
Dol m   AAVGNTRLVG NFIAMIAKKL VEQYKVPMTN IRLVGHSLGA HISGFAGKRV   150

Ves m   QELKLGKYSE IIGLDPARPS FDSNHCSERL CETDAEYVQI IHTSNYLGTE   200
Ves v   QELKLGKYSE IIGLDPARPS FDSNHCSERL CETDAEYVQI IHTSNYLGTE   200
Dol m   QELKLGKFSE IIGLDPAGPS FKKNDCSERI CETDAHYVQI LHTSSNLGTA   200

Ves m   KILGTVDFYM NNGKNNPGCG RFFSEVCSHT RAVIYMAECI KHECCLIGIP   250
Ves v   KTLGTVDFYM NNGKNQPGCG RFFSEVCSHS RAVIYMAECI KHECCLIGIP   250
Dol m   RTLGTVDFYI NNGSNQPGCR YIIGETCSHT RAVKYFTECI RRECCLIGVP   250

Ves m   RSKSSQPISR CTKQECVCVG LNAKKYPSRG SFYVPVESTA PFCNNKGKII   300
Ves v   KSKSSQPISS CTKQECVCVG LNAKKYPSRG SFYVPVESTA PFCNNKGKII   300
Dol m   QSKSPQPVSK CTRNECVCVG LNAKKYPKRG SFYVPVEAEA PYCNNNGKII   300
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FIG. 1. Amino acid sequences of two yellow jacket and one hornet phospholipase: Ves v 1, Ves m 1, and Dol m 1. *Underlined regions* of Ves v 1 represent sequences established by Edman degradation. Residues of Ves m 1 or Dol m 1 that differ from those of Ves v 1 are indicated by *dots.* Data for Dol m 1 and Ves m 1 are from Soldatova et al.[10] and Hoffman,[11] respectively.

amplify two cDNA fragments. One fragment encodes residue 1-59 together with a leader sequence of 153 nucleotides, and the other encodes residue 136-300 together with a 3′-untranslated region of 282 nucleotides. These three fragments were cloned into pCR plasmid, and two clones each of these fragments were sequenced. The combined data yielded a cDNA of 1341 nucleotides. Its sequence is not shown, but it is accessible through GENBANK, accession number L43561.

The amino acid sequence of Ves v 1 shown in Fig. 1 is obtained by translation of the cDNA. As noted previously, the cDNA includes a leader sequence, which is not shown in the translated protein sequence. The primary structure of Ves v 1 in Fig. 1 is supported by the partial sequence data from Edman degradation of the intact protein and its CNBr cleavage products (represented as underlined regions in Fig. 1). There is one exception, residue 1, which was shown to be phenylalanine according to Edman degradation of the natural protein but was found to be glycine according to the cDNA data. Thus residue 1 may be a polymorphic site. Also shown in Fig. 1 are the reported sequences of white-faced hornet phospholipase Dol m 1[10] and another yellow jacket phospholipase from *V. maculifrons,* Ves m 1.[11] The two yellow jacket phospholipases have 95% sequence identity, and they have about 67% identity with the hornet protein.

A cDNA encoding residue 1-300 of Ves v 1 was obtained from venom cDNAs by PCR with primers 10 and 11 in Table I. After restriction enzyme digestion, the cDNA was inserted into an expression vector pQE12. Bacteria were transformed with the recombinant plasmid; one clone was selected for expression, and it was found to contain the expected cDNA of Ves v 1 by DNA sequence analysis of the complete molecule. The recombinant protein has the protein sequence of MRGS-(Ves v 1)-SRH_6 and was obtained in a yield of about 7 mg per liter of culture.

On SDS electrophoresis recombinant (r) Ves v 1 showed one main band of 43 ± 2 kd together with other minor bands although the expected size is 35 kd and it is estimated to be of about 90% purity (Fig. 2, *A, lane 2*). The molecular size of rVes v 1 is greater than expected. This difference is probably due to its anomalous behavior on SDS gel electrophoresis, because DNA analysis of the recombinant plasmid indicated its sequence to be correct. Also shown in Fig. 2, *A,* for comparison, are natural (n) Ves v 1, nDol m 1, rDol m 1, and nPol a 1 in lanes 1, 3, 4, and 5, respectively. Their

main bands are, respectively, one band of 36 ± 2 kd, two bands of 38 and 36 kd, one band of 38 kd, and one band of 37 kd. The sample of nDol m 1 showed a single band of 38 kd when it was isolated, and the second band developed on storage. Vespid phospholipases are known to be susceptible to proteolysis.[6]

On immunoblotting, the main band of rVes v 1 did not react with mouse antibodies specific for nVes v 1 (data not shown), but it did react with antibodies specific for the reduced nVes v 1 (Fig. 2, *B*) and with antibodies specific for fragment M of hornet phospholipase Dol m 1, residue 102-221 (Fig. 2, *C*). Immunoblots in Fig. 2, *B* and *C*, were made with mercaptoethanol-reduced samples. Similar blots were made without prior reduction, and no binding of natural phospholipase-specific antibodies was observed (data not shown). The lack of binding of natural phospholipase for natural phospholipase-specific antibodies on immunoblotting suggests that there was denaturation of natural phospholipase during SDS gel electrophoresis.

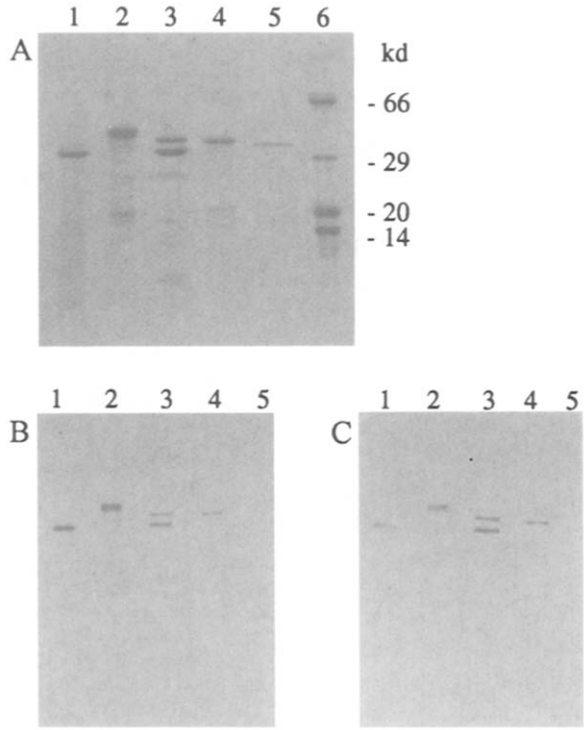

FIG. 2. SDS gel electrophoresis and immunoblot of reduced natural and recombinant vespid phospholipases. **A,** Dye-stained gel. **B and C,** Immunoblots probed with mouse sera specific for reduced nVes v 1 and for fragment M of nDol m 1, 102-221, respectively. *Lanes 1 and 2,* nVes v 1 and rVes v 1; *lanes 3 and 4,* nDol m 1 and rDol m 1; *lanes 5 and 6,* nPol a 1 and standards, respectively.

Expression of hornet phospholipase, Dol m 1, and its middle fragment

Recombinant Dol m 1 had not been expressed previously,[10] and it was expressed in this study by using a pQE8 vector. The necessary cDNA encoding Dol m 1 was obtained from venom cDNAs by PCR with primers 12 and 13 in Table I. One clone of the transformed bacteria was chosen for expression. Sequence analysis showed it to contain the expected cDNA sequence of Dol m 1[10] with one base change, which resulted in a Leu to Pro mutation at residue number 76. The recombinant protein from the pQE8 vector differs from that of the pQE12 vector mainly in the location of the hexahistidine tag. It has the sequence of MRGSH$_6$GS-(Dol m 1)-KLN. Recombinant Dol m 1 was obtained in a yield of about 7 mg per liter of bacteria culture. Its biochemical properties are similar to those of rVes v 1. It produced mainly one band of 38 kd on SDS gel electrophoresis (Fig. 2, *A, lane 4*), and the expected size is 35 kd. On immunoblot, it reacted strongly with antibodies specific for fragment M of Dol m 1, residue 102-221 (Fig. 2, *C*), and less strongly with antibodies specific for reduced nVes v 1 (Fig. 2, *B*), but not with antibodies specific for the nDol m 1 (results not shown).

Fragment M was expressed in bacteria with pQE12 vector, and the necessary cDNA was amplified with primers 14 and 15 in Table I. The recombinant fragment showed one band of the expected size of about 13 kd on gel electrophoresis, and it reacted strongly with antibodies specific for rVes v 1 on immunoblots (results not shown).

Cloning and expression of hyaluronidase Ves v 2

Partial amino acid sequence data showed sequence identity of Ves v 2 and Dol m 2 in the regions of residues 8-13 and 261-266. Two degenerate primers, 16 and 17 in Table II, corresponding to these two peptide regions, were used to amplify this segment of Ves v 2–specific cDNA from total venom by PCR. A cDNA encoding the full length of Ves v 2 and its 3′-untranslated region was obtained by 3′ RACE with primers 18 and 2 in Table I. These cDNAs were cloned into pCR plasmid. Two clones containing the full length of Ves v 2 and one clone encoding residue 8-266 of Ves v 2 were sequenced. The data yielded a cDNA of 1335 nucleotides, of which the first 993 nucleotides encode the Ves v 2 molecule. The DNA

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Dol m     SERPKRVF NIYWNVPTFM CHQYGLYFDE VTN FNIKHN SKDDFQGDKI    47
Ves v     SERPKRVF NIYWNVPTFM CHQYDLYFDE VTN FNIKRN SKDDFQGDKI    47
Api m   PDNNKTVREF NVYWNVPTFM CHKYGLRFEE VSEKYGILQN WMDKFRGEEI    50

Dol m   SIFYDPGEFP ALLPLKEGNY KIRNGGVPQE GNITIHLQRF IENLDKTYPN    97
Ves v   AIFYDPGEFP ALLSLKDGKY KKRNGGVPQE GNITIHLQKF IENLDKIYPN    97
Api m   AILYDPGMFP ALLKDPNGNV VARNGGVPQL GNLTKHLQVF RDHLINQIPD   100

Dol m   RNFNGIGVID FERWRPIFRQ NWGNMMIHKK FSIDLVRNEH PFWDKKMIEL   147
Ves v   RNFSGIGVID FERWRPIFRQ NWGNMKIHKN FSIDLVRNEH PTWNKKMIEL   147
Api m   KSFPGVGVID FESWRPIFRQ NWASLQPYKK LSVEVVRREH PFWDDQRVEQ   150

Dol m   EASKRFEKYA RLFMEETLKL AKKTRKQADW GYYGYPYCFN MSPNNLVPDC   197
Ves v   EASKRFEKYA RFFMEETLKL AKKTRKQADW GYYGYPYCFN MSPNNLVPEC   197
Api m   EAKRRFEKYG QLFMEETLKA AKRMRPAANW GYYAYPYCYN LTPNQPSAQC   200

Dol m   DATAMLENDK MSWLFNNQNV LLPSVYIRHE LTPDQRVGLV QGRVKEAVRI   247
Ves v   DVTAMHENDK MSWLFNNQNV LLPSVYVRQE LTPDQRIGLV QGRVKEAVRI   247
Api m   EATTMQENDK MSWLFESEDV LLPSVYLRWN LTSGERVGLV GGRVKEALRI   250

Dol m   SNNLKHS PK VLSYWWYVYQ DDTNTFLTET DVKKTFQEIA INGGDGIIIW   296
Ves v   SNNLKHS PK VLSYWWYVYQ DETNTFLTET DVKKTFQEIV INGGDGIIIW   296
Api m   ARQMTTSRKK VLPYYWYKYQ DRRDTDLSRA DLEATLRKIT DLGADGFIIW   300

Dol m   GSSSDVNSLS KCKRLREYLL TVLGPITVNV TETVN                   331
Ves v   GSSSDVNSLS KCKRLQDYLL TVLGPIAINV TEAVN                   331
Api m   GSSDDINTKA KCLQFREYLN NELGPAVKRI ALNNNANDRL TV          342
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FIG. 3. Amino acid sequences of yellow jacket, hornet, and honeybee hyaluronidases: Ves v 2, Dol m 2, and Api m 2. Data for Dol m 1 and Api m 1 are from Lu et al.[9] and Gmachl and Kreil,[22] respectively. *Underlined* regions of Ves v 2 were determined by protein sequencing.

TABLE II. ELISA of vespid natural phospholipase-specific mouse sera

Sera		Reciprocal titer on solid-phase natural or recombinant PLA				
Number	**Specificity**	**nDol m 1**	**rDol m 1**	**nVes v 1**	**rVes v 1**	**nPol a 1**
85W	nDol m 1	7×10^3	$<1 \times 10^1$	1×10^2	$<1 \times 10^1$	$<<1 \times 10^1$
Dol 83	nDol m 1	3×10^4	$<1 \times 10^1$	5×10^1	$<1 \times 10^1$	$<1 \times 10^1$
95K11	nDol m 1, reduced	5×10^2	1×10^3	ND	ND	ND
85I	nVes v 1	5×10^2	$<1 \times 10^1$	1×10^4	1×10^2	$<1 \times 10^1$
Ves83	nVes v 1	3×10^2	$<1 \times 10^1$	6×10^4	3×10^2	2×10^1
95K12	nVes v 1, reduced	ND	ND	5×10^3	5×10^3	ND
85K	nPol a 1	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	9×10^3
86K	nPol a 1	1×10^2	$<1 \times 10^1$	1×10^2	$<1 \times 10^1$	7×10^3
95K13	nPol a 1, reduced	ND	ND	ND	ND	2×10^3

PLA, Phospholipase A; *ND*, not done.

sequence is not given here, but it is accessible through GENBANK, accession number L43562.

In Fig. 3 the amino acid sequence of Ves v 2, which is from translation of the cDNA sequence described previously, is given; the underlined regions were also determined by protein sequencing. Residue 1 of Ves v 2 was found to be threonine by protein sequencing. Residue 1 was not determined by cDNA analysis as in 3′ RACE; the sense primer number 18 is from our earlier studies of Dol m 2, which has serine as its N-terminal residue. Also shown in Fig. 3 are the reported sequences of white-faced hornet and honeybee hyaluronidases, Dol m 2 and Api m 2.[9, 22] Yellow jacket and hornet hyaluronidases have 92% sequence identity with each other, and

they have 47% and 56% sequence identity with the bee protein, respectively.

For expression of Ves v 2, a cDNA encoding the full length of the molecule was obtained from venom cDNAs by PCR with primers 18 and 19 in Table I. The cDNA, after digestion with restriction enzyme, was inserted into the expression vector pQE8. The Ves v 2 insert in recombinant plasmid was verified by sequencing. Recombinant Ves v 2 was produced in bacteria transformed with the recombinant plasmid, and it was isolated in a yield of about 2 mg per liter of culture. It has the sequence of $MRGSH_6$-(Ves v 2)-KLN with a Thr to Ser substitution at residue 1.

On SDS gel electrophoresis, rVes v 2 produced mainly one band of about 41 kd, similar in size to those of nVes v 2, nDol m 2, rDol m 2, and nPol a 2 (Fig. 4, *A*). Recombinant Ves v 2 was contaminated with smaller digested fragments, and attempts to remove the fragments by changes in chromatographic conditions were not successful. We have previously observed the susceptibility of rDol m 2 to proteolysis.[9] On immunoblot, rVes v 2 reacted with antibodies specific for rVes v 2 or rDol m 2 (Fig. 4, *B* and *C*) and with antibodies specific for the natural proteins (results not shown). As described below, only some sera specific for natural hyaluronidase reacted with recombinant hyaluronidase.

The data from gel electrophoresis in Fig. 4 indicate that nVes v 2 is larger than rVes v 2. This difference is because nVes v 2 is a glycoprotein, as we have shown previously.[15]

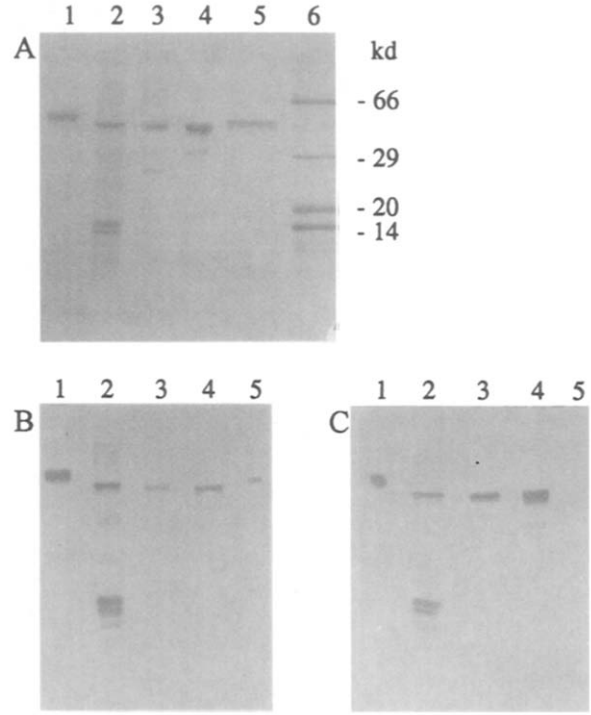

FIG. 4. SDS gel electrophoresis and immunoblot of reduced natural and recombinant vespid hyaluronidases. **A,** Dye-stained gel. **B and C,** Immunoblots probed with mouse sera specific for rVes v 2 and rDol m 2, respectively. *Lanes 1 and 2,* nVes v 2 and rVes v 2; *lanes 3 and 4,* nDol m 2 and rDol m 2; *lanes 5 and 6,* nPol a 2 and standards, respectively.

Murine antibody response to continuous and discontinuous epitopes of vespid allergens

Mouse sera specific for the vespid natural phospholipases or natural hyaluronidases, which were obtained after four or more immunizations, showed high antibody titers of 10^4 to 10^5 by ELISA on solid-phase natural allergen, but they produced low titers of 10^2 or less on solid-phase reduced or recombinant allergen. Sera specific for the reduced natural allergen or recombinant allergen showed high titers of 10^3 to 10^4 on solid-phase recombinant allergen and usually lower titers on solid-phase natural allergen. ELISA data of sera specific for natural phospholipases or their reduced proteins are given in Table II. Similar data for hyaluronidase-specific sera are not shown.

All the serum samples used in this study are pools obtained from three to four mice. The majority of serum samples were obtained from mice immunized with alum adjuvant. Four serum samples were from a previous study[13] in which complete Freund's adjuvant was used with mice immunizations: sera Dol83 and Ves83 in Table II and sera Ves82 and Pol83 in Table III. Thus our findings are not likely to be due to individual animal variation or to the use of alum or complete Freund's adjuvant.

These results are similar to our previous findings with natural and recombinant hornet antigen 5,[23] and they are related to the specificity of antibodies for the continuous and discontinuous B-cell epitopes. These data suggest that more than 90% of the vespid natural phospholipase- or natural-hyaluronidase-specific antibodies recognize their discontinuous epitopes, as we have shown for vespid antigen 5s.

Antigenic cross-reactivity of vespid allergens tested with mouse antibodies

We had reported previously that cross-reactions of vespid natural phospholipases by inhibition analysis were not detectable by inhibition ELISA

TABLE III. Summary of immunoblot results of reduced vespid allergens on probing with sera of different specificities[1]

Sera		Reduced vespid phospholipases		
Number	**Specificity**	**nDol m 1**	**nVes v 1**	**nPol a 1**
85W	nDol m 1	−	−	ND
Dol83	nDol m 1	−	−	ND
95K05	nDol m 1	+	−	ND
95K11	nDol m 1, reduced	++	+	−
95K06*	rDol m 1, frag M	++	+	−
85K	nVes v 1	−	−	ND
Ves83	nVes v 1	−	+	ND
94K22	rVes v 1	+	++	ND
95K12*	nVes v 1, reduced	+	++	±
85K	nPol a 1	−	−	−
95K13	nPol a 1, reduced	+	+	++
		Reduced vespid hyaluronidases		
		nDol m 2	**nVes v 2**	**nPol a 2**
94K09	nDol m 2	−	−	−
93K15	nDol m 2	+	−	−
95K14†	rDol m 2	++	+	−
Ves82	nVes v 2	+	++	+
95K15†	rVes v 2	++	++	+
Pol83	nPol a 2	−	−	−

Relative intensities of bands are ranked as ++, +, ± and −.
ND, Not done.
*Blots probed with these sera are shown in Fig. 2.
†Blots probed with these sera are shown in Fig. 4.

with specific mouse sera, but partial cross-reactions of vespid natural antigen 5s and natural hyaluronidases were detectable by this method.[13] When natural phospholipase-specific mouse sera were tested by the more sensitive direct ELISA for their binding to solid-phase autologous or homologous protein, a low level of cross-reactivity was detected with two serum samples specific for each phospholipase (Table II). The antibody titer for the binding of hornet- or yellow jacket–specific sera to homologous phospholipases is several hundred fold less than its binding to the autologous protein. One of the two wasp phospholipase-specific sera (sera 86K in Table II) showed a weak binding of hornet or yellow jacket protein, but the other (sera 85K) did not.

ELISA data for antigen 5– and hyaluronidase-specific mouse sera were also obtained (results not shown). In accord with the previous inhibition ELISA data, vespid antigen 5s or hyaluronidases showed a higher degree of cross-reactivity than the phospholipases.

Immunoblots of reduced vespid allergens were used to estimate the extent of cross-reactivity of their continuous B-cell epitopes. These results were obtained with 11 serum samples of different specificity for phospholipases, and they are summarized in Table III; the results for sera 95K06 and 95K12 and for sera 95K14 and 95K15 are also shown in Figs. 2 and 4, respectively. Cross-reactivity of hornet and yellow jacket phospholipases was only detectable with one of the five natural phospholipase-specific sera tested but was routinely detectable with all four sera specific for recombinant phospholipase or reduced natural phospholipase. Cross-reactivity of hornet or yellow jacket phospholipase with wasp protein was detectable with two of three serum samples specific for reduced protein, 95K12 and 95K13.

Cross-reactivity of three vespid hyaluronidases was detectable with three of the six sera tested (Table III). We have shown previously that cross-reactivity of vespid antigen 5s was detectable with all sera tested.

These data on the continuous and the discontinuous B-cell epitopes of vespid hyaluronidases and phospholipases both indicate a greater degree of cross-reactivity among hornets and yellow jackets

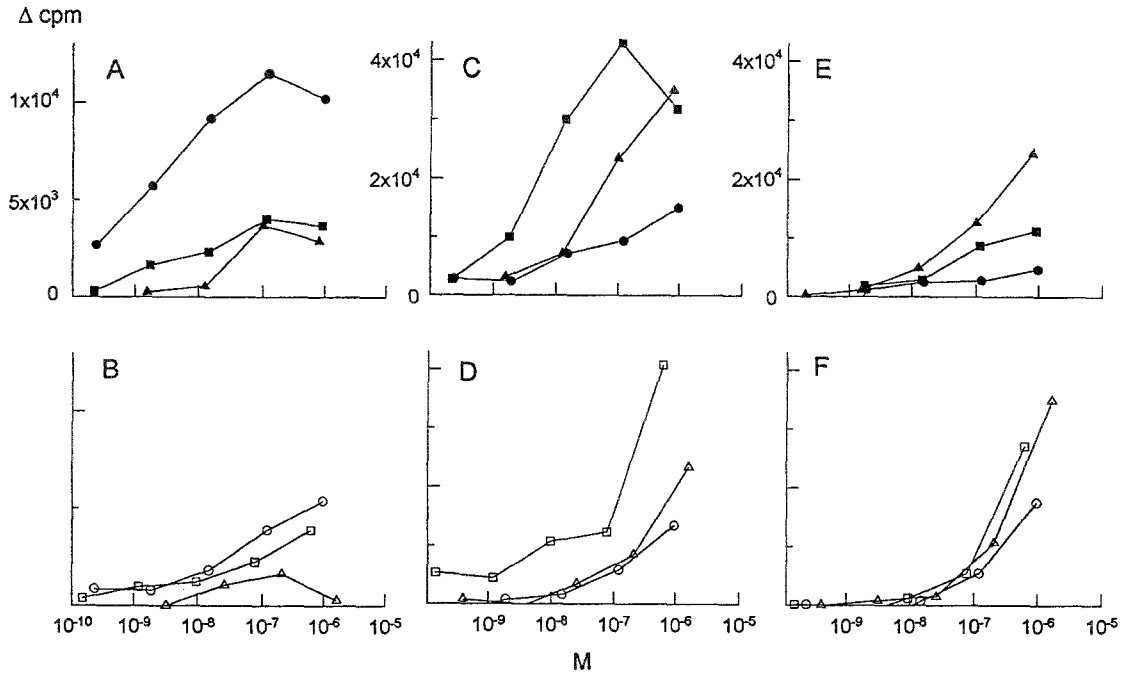

FIG. 5. Proliferation assay with spleen cells from mice immunized with reduced vespid phospholipases. Natural Dol m 1–, nVes v 1–, and nPol a 1–specific spleen cells were used in **A and B, C and D,** and **E and F,** respectively. Stimulating antigens are nDol m 1 (●), nVes v 1 (■), nPol a 1 (▲), and their respective reduced derivatives are represented by open symbols. Background proliferations were 4100, 8200, and 8300 cpm in these respective graphs.

than that among hornets or yellow jackets and wasps.

Antigenic cross-reactivity of vespid phospholipases and hyaluronidases tested by proliferation assay with specific mouse spleen cells

To study the cross-reactivity of the T-cell epitopes of vespid phospholipases, spleen cells from mice immunized with the reduced natural proteins were used. In Fig. 5, *A* and *B,* the proliferation results of Dol m 1–specific spleen cells on stimulation with the three vespid natural phospholipases and their reduced natural proteins are given, respectively. In Fig. 5 (*C* and *D*, and *E* and *F*) the similar results of Ves v 1– and Pol a 1–specific spleen cells are given, respectively.

The proliferation data in Fig. 5 show that greater maximal stimulation of vespid phospholipase-specific spleen cells was obtained by the autologous protein than by the homologous proteins. In each case the concentration of natural protein required for maximal stimulation is about 10-fold lower than that of its reduced natural protein. Recombinant Dol m 1 and rVes v 1 were also tested as stimulating antigens, and they produced results similar to those for their reduced natural proteins (data not shown). The results shown in Fig. 5 suggest the partial cross-reactivity of the T-cell epitopes of vespid phospholipases. In contrast to the data for B-cell epitopes, these data also suggest a greater degree of cross-reactivity of the yellow jacket Ves v 1 and the wasp Pol a 1 than that of the yellow jacket Ves v 1 and the hornet Dol m 1.

In Fig. 6, *A* and *B,* the proliferation results of hornet rDol m 2–specific spleen cells, on stimulation with the natural vespid hyaluronidases and their recombinant proteins are given, respectively. As shown in Fig. 6, *A,* nDol m 2 and nPol a 2 were equally effective in their stimulation of rDol m 2–specific spleen cells, and surprisingly, nVes v 2 was more effective than nDol m 2. But as shown in Fig. 6, *B,* rDol m 2 appeared to be a more effective stimulating antigen than rVes v 2. The heteroclitic response of rDol m 2–specific spleen cells to nVes v 2 was not observed in a separate experiment with nDol m 2–specific spleen cells (results not shown).

In Fig. 6, *C* and *D,* the proliferation results of yellow jacket rVes v 2–specific spleen cells on stimulation with the natural vespid hyaluronidases and their recombinant proteins are given, respectively. The findings are similar to those shown in

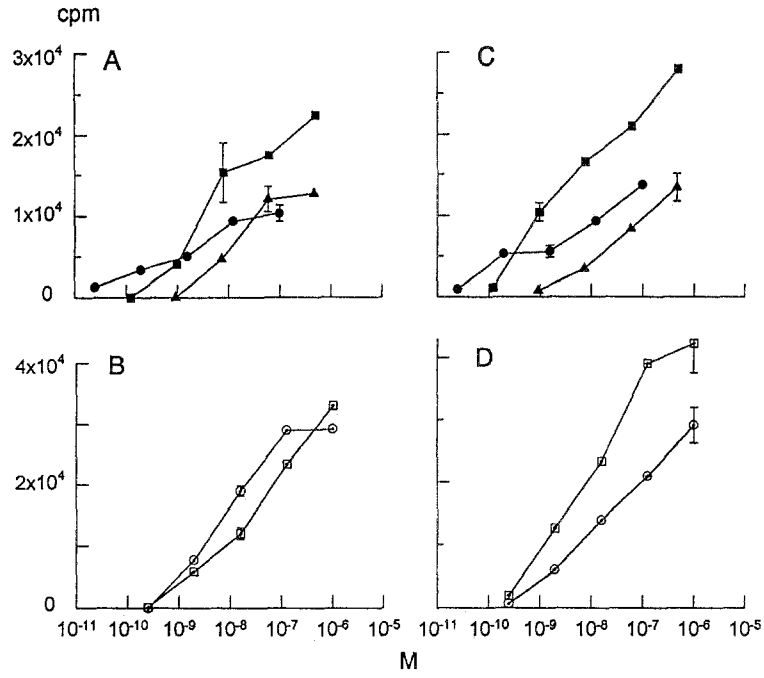

FIG. 6. Proliferation assay with spleen cells from mice immunized with recombinant hornet and yellow jacket hyaluronidases. Recombinant Dol m 2– and rVes v 2–specific spleen cells were used in **A and B** and **C and D**, respectively. Stimulating antigens are nDol m 2 and rDol m 2 (● and ○, respectively), nVes v 2 and rVes v 2 (■ and □, respectively) and nPol a 2 (▲). Background proliferations were 7500 ± 500 cpm in these graphs.

Fig. 6, *A* and *B*. These findings together indicate that there is partial cross-reactivity of the T-cell epitopes of vespid hyaluronidases.

DISCUSSION

We have compared antigenic cross-reactivities of two venom allergens, hyaluronidase and phospholipase A_1 from hornets, wasps, and yellow jackets in mice in this report. These findings together with our earlier findings on another vespid venom allergen, antigen 5, indicate that the homologous venom allergens from these three vespids share limited cross-reactivities of their B- and T-cell epitopes. Furthermore, these venom allergens differ in the cross-reactivities of their continuous and discontinuous B-cell epitopes.

The degree of cross-reactivity of the continuous B- and T-cell epitopes of vespid allergens is limited because in no case does the homologous antigen behave identically to the autologous antigen in binding of specific antibodies (Table III) or in stimulation of specific spleen cells (Figs. 5 and 6). Some or all of the cross-reacting linear epitopes are likely to have very similar or identical amino acid sequences because the homologous venom allergens have 70% to 90% sequence identity (Figs. 1 and 3). A weak cross-reactivity of the discontinuous B-cell epitopes of homologous vespid antigen 5s and hyaluronidases is detectable by inhibition ELISA analysis,[13] as well as by the more sensitive direct ELISA. Cross-reactivity of the homologous vespid phospholipases is not detectable by inhibition ELISA, but it is detectable by direct ELISA (Table II).

It is of interest to note that the homologous antigen 5s and phospholipases of hornets and yellow jackets have almost the same extent of sequence identity (69% and 67%, respectively), yet they differ markedly in the cross-reactivity of their discontinuous B-cell epitopes but not of their continuous epitopes. Also, we may note that vespid hyaluronidases have about 53% sequence identity with bee hyaluronidase (Fig. 3) and that antigenic cross-reactivity of these proteins had been detected by RAST inhibition with patient sera.[24, 25] These findings are to be expected, because the degree of antigenic cross-reactivity of homologous proteins depends on the local sequence identity of the epitopes of interest.

Our tests in the murine system show that with all three vespid allergens, there is a greater degree of cross-reactivity among the homologous allergens

of hornets and yellow jackets than among the homologous allergens of hornets or yellow jackets and wasps. This is the same order as that reported for the frequency of patients' multiple sensitivity to these insects.[1] Our observation that more than 90% of vespid allergen-specific murine antibodies recognize their discontinuous B-cell epitopes may also be the case in human beings.

The exact mechanism of protection after venom immunotherapy is not yet known. It could be due to the rise of venom-specific IgGs with accompanying decreases in venom-specific IgEs,[26] the downregulation of the venom-specific T-cell responses with accompanying changes in cytokine release,[27] or both. The cytokines influence not only the Ig class switch events of activated B cells but also the inflammatory responses of allergic conditions.

With the knowledge of antigenic cross-reactivity of vespid venoms, some physicians attempt to treat patients with sensitivity to multiple vespids by immunotherapy with a single vespid venom.[28] Others have reported that treatment of patients sensitive to yellow jackets with mixed venoms provides better clinical and IgG antibody responses than treatment with yellow jacket venom alone.[5, 29]

Our findings in mice indicate that the three vespid allergens each show varying extents of limited cross-reactivity of their B-cell epitopes, in particular their discontinuous B-cell epitopes. Immunotherapy of patients with sensitivity to multiple vespids with a single vespid venom can only induce antibodies specific for the immunizing venom, and these antibodies will not cross-react fully with the other vespid venoms. Thus treatment with a single venom may not provide adequate protection if the protective mechanism of immunotherapy is primarily due to changes in allergen-specific antibody levels.

However, immunotherapy of patients with a single vespid venom may lead to downregulation of T cells specific for the immunizing venom, as well as those for cross-reacting venoms. Our studies in mice indicate partial cross-reactivities of the T-cell epitopes of homologous vespid allergens. These partial cross-reactivities may be a consequence of substitution of amino acid residues, which are involved in the binding of the antigenic peptide–major histocompatibility complex to the T-cell receptor. Binding of such a cross-reacting venom peptide complex to the receptor can nonetheless be still active in inducing T-cell unresponsiveness.[30] Therefore if downregulation of allergen-specific T-cell responses is an important factor in the mechanism of immunotherapy, it might be useful to treat patients with a cross-reacting venom. This would be advantageous because the cross-reacting venom would have lower allergenicity than the venom to which the patient is sensitive, and thus it could be used at a higher dose and possibly with greater safety.

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REFERENCES

1. Hoffman D, Miller J, Sutton J. Hymenoptera venom allergy: a geographic study. Ann Allergy 1980;45:276-9.
2. Reisman R, Wypych J, Mueller U, Grant J. Comparison of the allergenicity and antigenicity of Polisters venom and other vespid venoms. J Allergy Clin Immunol 1982;70: 281-7.
3. Hoffman D. Allergens in Hymenoptera venom. VI. Cross reactivity of human IgE antibodies to the three vespid venoms and between vespid and paper wasp venoms. Ann Allergy 1981;46:304-9.
4. Reisman RE, Mueller U, Wypych J, Elliott W, Arbesman C. Comparison of the allergenicity and antigenicity of yellowjacket and hornet venoms. J Allergy Clin Immunol 1982;69:268-74.
5. Golden DBK, Valentine MD, Kagey-Sabotka AK, Lichtenstein LM. Cross-reactivity of vespid venoms [abstract]. J Allergy Clin Immunol 1981;67:57.
6. King TP, Sobotka A, Alagon A, Kochoumian L, Lichtenstein LM. Protein allergens of white-faced hornet, yellow hornet and yellowjacket venoms. Biochemistry 1978;17: 5165-74.
7. Lu G, Villalba M, Coscia MR, Hoffman DR, King TP. Sequence analysis and antigen cross reactivity of a venom allergen antigen 5 from hornets, wasps and yellowjackets. J Immunol 1993;150:2823-30.
8. Hoffman DR. Allergens in Hymenoptera venom XXV: the amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity. J Allergy Clin Immunol 1993;92:707-16.
9. Lu G, Kochoumian L, King TP. Sequence identity and antigenic cross reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. J Biol Chem 1995;270:4457-65.
10. Soldatova L, Kochoumian L, King TP. Sequence similarity of a hornet *(D. maculata)* venom allergen phospholipase A1 with mammalian lipases. FEBS Lett 1993;320:145-9.
11. Hoffman DR. Allergens in Hymenoptera venom XXVI: the complete amino acid sequences of two vespid venom phospholipases. Int Arch Allergy Immunol 1994;104:184-90.
12. Hoffman DR. The structure of vespid phospholipases and the basis for their immunologic crossreactivity [abstract]. J Allergy Clin Immunol 1994;93:223.

13. King T, Joslyn A, Kochoumian L. Antigenic cross-reactivity of venom proteins from hornets, wasps and yellowjackets. J Allergy Clin Immunol 1985;75:621-8.
14. King T, Kochoumian L, Joslyn A. Wasp venom proteins: phospholipase A1 and B. Arch Biochem Biophys 1984;230: 1-12.
15. King TP, Alagon AC, Kuan J, Sobotka AK, Lichtenstein LM. Immunochemical studies of yellowjacket venom proteins. Mol Immunol 1983;20:297-308.
16. Fang KSF, Vitale M, Fehlner P, King TP. cDNA cloning and primary structure of a white-face hornet venom allergen, antigen 5. Proc Natl Acad Sci USA 1988;85:895-9.
17. Frohman MA. Rapid amplification of cDNA ends. Amplications Forum PCR Users. 1990;5:11-5.
18. Frohman MA, Dush MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 1988;85:8998-9002.
19. Barnard R, Southard JN, Talamantes F. Two-step PCR amplification of multiple specific products from cDNA using one specific primer and oligo dT. BioTechniques 1994;16:169-70.
20. Clark JM. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. Nucl Acids Res 1988;16:9677-86.
21. Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
22. Gmachl M, Kreil G. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. Proc Natl Acad Sci USA 1993;90:3569-73.
23. King TP, Kochoumian L, Lu G. Murine T and B cell responses to natural and recombinant hornet venom allergen, Dol m 5.02 and its recombinant fragments. J Immunol 1995;154:577-84.
24. Wypch J, Abenounis C, Reisman R. Analysis of differing patterns of cross-reactivity of honey bee and yellowjacket venom-specific IgE: use of purified venom fractions. Int Arch Allergy Appl Immunol 1989;89:60-6.
25. Hoffman D, Wood C. Allergens in Hymenoptera venom XI. Isolation of protein allergens from *Vespula maculifrons* (yellowjacket) venom. J Allergy Clin Immunol 1984;74:93-103.
26. Golden D, Meyerd D, Kagey-Sobotka A, Valentine M, Lichtenstein L. Clinical relevance of the venom-specific immunoglobulin G antibody level during immunotherapy. J Allergy Clin Immunol 1982;69:489-93.
27. Norman PS. Modern concepts of immunotherapy. Curr Opin Immunol 1993;5:968-73.
28. Reisman RE. Insect stings. N Engl J Med 1994;331:523-7.
29. Valentine MD. Samter's immunologic diseases. In: Frank MM, Austen KF, Claman HN, Unanue ER, editors. Insect venom allergy. Boston: Little, Brown & Co., 1995;2:1367-75.
30. Sloan-Lancaster J, Allen PM. Significance of T-cell stimulation by altered peptide ligands in T cell biology. Curr Opin Immunol 1995;7:103-9.