

Sequence Identity and Antigenic Cross-reactivity of White Face Hornet Venom Allergen, Also a Hyaluronidase, with Other Proteins*

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Gang Lu, Loucia Kochoumian, and Te Piao King

From The Rockefeller University, New York, New York 10021-6399

White face hornet (*Dolichovespula maculata*) venom has three known protein allergens which induce IgE response in susceptible people. They are antigen 5, phospholipase A₁, and hyaluronidase, also known as Dol m 5, 1, and 2, respectively. We have cloned Dol m 2, a protein of 331 residues. When expressed in bacteria, a mixture of recombinant Dol m 2 and its fragments was obtained. The fragments were apparently generated by proteolysis of a Met-Met bond at residue 122, as they were not observed for a Dol m 2 mutant with a Leu-Met bond.

Dol m 2 has 56% sequence identity with the honey bee venom allergen hyaluronidase and 27% identity with PH-20, a human sperm protein with hyaluronidase activity. A common feature of hornet venom allergens is their sequence identity with other proteins in our environment. We showed previously the sequence identity of Dol m 5 with a plant protein and a mammalian testis protein and of Dol m 1 with mammalian lipases.

In BALB/c mice, Dol m 2 and bee hyaluronidase showed cross-reactivity at both antibody and T cell levels. These findings are relevant to some patients' multiple sensitivity to hornet and bee stings.

Insect sting allergies to bees and vespids are common; the vespids include hornets, yellow jackets, and wasps (1, 2). Susceptible people can be sensitized on exposure to minute amounts of venom proteins, as <10 µg of protein is injected into the skin on a single sting (3). Insect venoms are used widely for the diagnosis and treatment of insect allergy (1, 2). Patients are screened for the presence of specific IgEs by skin tests with venom and are protected against subsequent stings by subcutaneous injections of increasing amounts of venom. On treatment with venom, patients show a rapid rise of specific IgEs and IgGs followed by a gradual decrease of IgEs. Recent studies suggest that immunotherapy down-regulates allergen-specific T cell responses, thus altering the allergen-specific antibody response (4).

Insect allergy, like other forms of immediate-type allergy, e.g. hay fever, results from interaction of cell-bound specific IgEs with allergens. Immune response, irrespective of the antibody isotype, is known to be controlled by the genetic make-up of the host, the route and the mode of immunization, and the nature of the immunogen. It is not clear to what extent the nature of the immunogen, or allergen, determines the extent of IgE response in susceptible people.

The vespids have similar venom compositions. They each

contain a homologous set of three major venom allergens: antigen 5 of unknown biological function, hyaluronidase, and phospholipase A₁ (5). Antigens 5 from several species of different vespids have been cloned or sequenced and are found to be proteins of 203–205 amino acid residues (6). Phospholipases A₁ from white face hornet (*Dolichovespula maculata*) (7) and two species of yellow jackets (*Vespula maculifrons* and *vulgaris*) (8, 9) have been cloned or sequenced and are found to be proteins of 300 amino acid residues.

The enzymatic specificity of vespid hyaluronidase is of the endo-*N*-acetylhexosaminidase type (10), as it catalyzes the release of reducing groups of *N*-acetylglucosamine from hyaluronic acid, a polymer of repeating disaccharides of D-glucuronic acid and *N*-acetyl-D-glucosamine. In this paper we will report our findings on the cloning and expression of white face hornet hyaluronidase, its sequence similarity with the bee venom allergen hyaluronidase (11) and a mammalian testis protein known as PH-20 (12, 13), and its antigenic cross-reactivity with bee venom hyaluronidase. Human PH-20 was recently reported to have hyaluronidase activity (14). The three venom proteins of white face hornet (*D. maculata*), antigen 5, phospholipase A₁, and hyaluronidase, are designated as Dol m 5, 1, and 2, respectively, according to an accepted nomenclature system for allergens (15).

MATERIALS AND METHODS

Isolation and Partial Amino Acid Sequences of Hornet Hyaluronidase, Dol m 2—The desired protein was isolated from white face hornet (*D. maculata*) venom sac extract (Vespa Lab, Spring Mills, PA) as described previously (5). Briefly, the venom sac extract was first depleted of its phospholipase A₁ component (Dol m 1) by affinity chromatography on an adsorbent containing a substrate analog of phospholipase followed by affinity chromatography on heparin Sepharose to separate antigen 5 (Dol m 5) and hyaluronidase (Dol m 2) (16). 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.).

cDNA of Dol m 2—Total RNAs were isolated from venom acid glands of *D. maculata* (17). Dol m 2-specific cDNA was obtained from total RNAs by Frohman's procedure (18, 19) for rapid amplification of 3'- or 5'-cDNA ends (RACE).¹

First strand cDNAs for 3'-RACE were synthesized from the MeHgOH denatured total RNAs (5 µg) as the template, with oligonucleotide number 5 (Table I) as the primer, using the cDNA synthesis system from Life Technologies, Inc. For 5'-RACE, the single-stranded cDNAs were synthesized as described above, except that oligonucleotide number 8 (Table I) was used as the primer, then poly(dA)-tailed with terminal deoxynucleotidyltransferase (U. S. Biochemical). 3'- or 5'-RACE was carried out with GenAmp PCR reagent kit (Perkin-Elmer Cetus) using Amplitaq polymerase. PCR was carried out for two successive rounds of 30 cycles as follows: template, 2 µM each sense and antisense primers, 400 µM each dNTPs, and 5 units of *Taq* polymerase

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L34548.

¹ The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; r, recombinant.

(Perkin-Elmer Cetus) in 50 μ l of 10 mM Tris-HCl (pH 8.4) + 50 mM KCl + 1.5 mM MgCl₂. Each cycle consisted of 45 s at 94 °C, 25 s at 50 °C, and 3 min 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 reaction mixture.

PCR products were examined by electrophoresis in agarose gel with ethidium bromide staining and by Southern blot analysis where 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.). Plasmid DNAs were isolated using the Wizard Miniprep system (Promega Biotec). DNA sequences were determined by the dideoxynucleotide chain termination method using alkaline-denatured plasmid DNAs and the Sequenase Version 2.0 kit (U. S. Biochemical).

Expression of r-Dol m 2 with Ile to Phe Mutation at Residue 47—The necessary cDNAs were obtained by PCR amplification of a modified pCR plasmid template, clone 12, containing Dol m 2 cDNA in Table II. The necessary primers, numbers 11–13, are listed in Table I. The sense and antisense 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 μ g) was digested in 60 μ l of universal buffer with 20 units each of restriction enzymes *Bam*HI and *Bgl*II or *Hind*III (Life Technologies, Inc.) at 37 °C overnight. The product was isolated by electrophoresis in 1.4% LGT agarose (FMC). The gel slice containing the cDNA of interest was cut out, and 2–4 μ l of the melted gel (40–80 fmol of cDNA) was used for ligation with 18 ng (8 fmol) of appropriately cut and dephosphorylated pQE-12 or -8 vector (QIAGEN). Ligation was carried out overnight at 15 °C in the presence of 2 units of T4 DNA ligase in 60 μ l of buffer as supplied by the manufacturer (Life Technologies, Inc.).

Competent M15 [pREP4] cells were transformed with the above ligation mixture, then grown on LB-agar plates containing 25 μ g/ml kanamycin and 100 μ g/ml ampicillin as described by the manufacturer (QIAGEN). Selected colonies were screened by SDS-gel electrophoresis for expression of the expected protein after induction with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 1.5 h.

Positive colonies were grown in a culture of 250-ml volume. The collected bacteria pellet was washed free of culture with buffer of 0.05 M Tris-HCl (pH 8.0) + 0.10 M NaCl + 1 mM EDTA, then dissolved in 6 ml of 6 M guanidine hydrochloride + 0.10 M Tris-HCl (pH 8.0) + 20 mM 2-mercaptoethanol and heated to 100 °C for 5 min. After clarification by high speed centrifugation, the solution was applied to a 0.8 $\times$ 3.5 cm column of nickel-nitrilotriacetic acid agarose (QIAGEN). The column was washed in succession with 6-ml portions of 6 M urea + 20 mM 2-mercaptoethanol buffered at pH 8.0, 6.3, 5.9, and 4.5. r-Dol m 2 and its fragments were eluted with 6 M urea buffered with 0.14 M NH₄Ac at pH 4.5.

r-Proteins were freed of urea and buffer salts by chromatography on a 8 $\times$ 0.9 cm column of C18 silica (Separation Group, Hesperia, CA) with a linear 2-propanol gradient of 0.5% per ml in 0.1% trifluoroacetic acid. The r-proteins were eluted at about 40% 2-propanol. After lyophilization, they were found to be soluble in 0.02 N 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.

Expression of r-Dol m 2 with Met to Leu Mutation at Residue 122—Site-directed mutagenesis was made by the PCR overlap extension method (21). Double-stranded cDNAs encoding residues 1–125 and 119–331 were obtained by PCR amplification of hornet venom first strand cDNAs using two primer pairs, numbers 11 and 15 and numbers 14 and 13 of Table I. Primers 14 and 15 were designed so that the single base substitution resulted in a codon change from Met to Leu at residue 122.

Full length cDNA encoding the mutated Dol m 2 was obtained by PCR amplification of a mixture of mutated cDNAs encoding 1–125 and 119–331 using primers 11 and 13. The resulting PCR product was digested with *Bam*HI and *Hind*III, then ligated with appropriately cut and dephosphorylated pQE8. The remaining steps were the same as those described above.

Characterization of r-Dol m 2—SDS slab gel electrophoresis was made in 12.5% polyacrylamide gel (22). Samples were reduced by boiling in sample buffer + 1% 2-mercaptoethanol for 5 min prior to electrophoresis. For staining with Coomassie Blue R-250, about 4 μ g of each protein sample was used. For immunoblot, about 400 ng of each sample was used. The proteins in polyacrylamide gel were electroblotted onto nitrocellulose paper in a Model TE 70 Semi-Phor transfer unit (Hoeffer

Scientific Instruments) following the manufacturer's directions. The nitrocellulose paper was kept for 0.5 h in diluent buffer (see below), then in succession for 1-h periods with 1/300 diluted mouse antisera specific for natural Dol m 2, or for r-Dol m 2 with Ile to Phe mutation at residue 47, and 1/100 diluted sheep anti-mouse IgG conjugated with horseradish peroxidase (Sigma). The mouse sera were diluted in diluent buffer containing bacterial lysate (see below). The blots were stained in a substrate solution of 1.5 mg/ml 4-chloronaphthol and 0.01% H₂O₂ in 0.05 M Tris-HCl (pH 8.0).

Attempted Refolding of Recombinant Dol m 2—These experiments were made at a protein concentration of about 40 μ g/ml in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 0 or 2.5 mM 2-mercaptoethanol, and 0, 1, or 2 M guanidine hydrochloride. At time intervals of 0–72 h, aliquots were taken for the assay of its activity to depolymerize hyaluronic acid from human umbilical cord (Sigma) as measured by turbidity change (23). The sample of natural hyaluronidase which was used as a control had a specific activity of 3900 $\pm$ 250 units/mg.

Immunization and Immunoassays—Groups of 4 female BALB/c mice at 8–10 weeks of age (The Jackson Laboratories) were immunized intraperitoneally each with 0.2 ml of 10 μ g/ml bee or hornet hyaluronidase + 5 mg/ml alum in 0.05 M sodium phosphate (pH 6.4) on week 0, 2, 4, 6, and 8. Sera were collected by retro-orbital puncture 1 week after each immunization. Bee hyaluronidase was isolated from venom as described (24). The sample of recombinant hornet hyaluronidase used for immunogen contains an Ile to Phe mutation at residue 47.

Antibodies were measured by enzyme-linked solid-phase immunoassay. Microtiter wells were coated overnight with 5 μ g/ml antigen in 0.05 M 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 M NaCl, and 0.05 M Tris-HCl (pH 8.0). The wells were then treated for 1-h periods in succession with varying concentrations of mouse sera to be tested, 10 μ g/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% H₂O₂ in 0.1 M sodium phosphate (pH 7.0). Antibody titers were expressed as reciprocal dilutions required to give an absorbance change of 1.0 in 30 min.

Antigen and antibody solutions used above were prepared in diluent buffer. For enzyme-linked immunosorbent assay on solid-phase recombinant protein, the diluent buffer contained, in addition to the ingredients given earlier, 10% bacterial lysate. This lysate was prepared by sonication of bacteria in phosphate-buffered saline (containing 2 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, and 1 mM 2-mercaptoethanol) in 1/10 of the original culture volume, and the suspension was clarified by centrifugation before use. Addition of bacterial lysate was necessary to reduce background color development which might be due to the presence of antibodies specific for bacterial proteins in the reagents used.

Proliferation assays were made with spleen cells from two mice, 10 days after week 2 or week 8 immunizations. Spleen cells (2×10^5) were cultured with varying concentrations of test antigen in 0.2 ml of culture medium at 37 °C and 5% CO₂. [³H]Thymidine (1 μ Ci) was added on day 3, and the uptake of thymidine was counted on day 4. The culture medium consisted of RPMI 1640 (Life Technologies, Inc.), 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES buffered at pH 7.3 (Sigma), and 54 μ M 2-mercaptoethanol.

RESULTS

Cloning of Hornet Hyaluronidase, Dol m 2—Partial amino acid sequence data were obtained by Edman degradation of the intact protein and its *Staphylococcus aureus* protease-digested peptides. Two degenerate oligonucleotides 1 and 2 (Table I) were synthesized on the basis of partial amino acid sequence data and used as primers in the polymerase chain reaction (PCR) to amplify, from venom cDNAs, the cDNA which is encoded by these primers. The location of oligonucleotide 1 in the protein sequence was known; it encodes residues 8–13 of hyaluronidase. That of oligonucleotide 2 was established by comparison of the translated sequence of the PCR product with the partial amino acid sequence data of hyaluronidase; it encodes residues 40–45.

From the DNA sequence data encoding residues 8–45 of

TABLE I
Oligonucleotide primers for cloning of hornet hyaluronidase

No.	Primer	Notes
1.	CGT GGA TCC TTC AAC/T ATI TAC/T TGG AA F ⁸ N I Y W N ¹³	PCR for residues 8–45
2.	CGT AGA TCT TC ICC T/CTG A/GAA A/GTC A/GTC D ⁴⁵ G Q F D D ⁴⁰	See above
3.	TGG AAC GTT CCT ACC TTT ATG W ¹² N V P T F M ¹⁸	First round 3'-RACE
4.	GGC CTA TAC TTC GAC GAG G ²³ L Y F D E ²⁸	Second round 3'-RACE
5.	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
6.	AAG GAT CCG TCG ACA TC	First round 3'-RACE or second round 5'-RACE
7.	GAC ATC GAT AAT ACG AC Y ¹⁸² G Y Y G W ¹⁷⁷	Second round 3'-RACE
8.	G ATA TCC GTA ATA GCC CC D ¹⁰⁷ I V G I G ¹⁰²	cDNA synthesis of 5'-RACE
9.	TC GAT CAC ACC GAT ACC G L ⁶² P L L A P ⁵⁷	First round 5'-RACE
10.	AG CGG CAA CAA TGC CGG G S ¹ E R P K R ⁶	Second round 5'-RACE and sequencing primer
11.	CGT GGA TCC GAG AGA CCG AAA AGA N ³³¹ V T E T V ³²⁶	PCR for residues 1–331
12.	CGT AGA TCT GTT GAC GGT TTC CGT CAC N ³³¹ V T E T V ³²⁶	See above
13.	CGT AAG CTT GTT GAC GGT TTC CGT CAC W ¹¹⁹ G N L M I H ¹²⁵	See above
14.	TGG GGC AAT CTG ATG ATT CAT H ¹²⁵ I M L N G W ¹¹⁹	M ¹²² → L ¹²² mutagenesis
15.	ATG AAT CAT CAG ATT GCC CCA	See above

hyaluronidase, additional oligonucleotide primers 3 and 4 (Table I) were synthesized. They were used together with oligonucleotide primers 6 and 7 (Table I) to amplify the 3' ends of the cDNA encoding hyaluronidase by the 3'-RACE procedure (18, 19). In this manner, a cDNA fragment containing nucleotides 127–1229 (Fig. 1) was obtained. Another set of primers, 8–10 (Table I), was synthesized based on the DNA sequence data of 3'-RACE. They were used together with primers 5 and 6 to amplify the 5' end of the cDNA, and the cDNA fragment containing nucleotides 1–245 was obtained.

The N-terminal sequence of hyaluronidase for residues 1–45, which was deduced by Edman degradation, is encoded by nucleotide position 61–195 in Fig. 1. The region of nucleotide position 1–60 probably encodes a portion of the prepro segment of hyaluronidase. However, the presence of a stop codon at nucleotide position 19–21 is unexpected and may possibly represent incomplete splicing of mRNA. The coding region of the DNA in Fig. 1 ends at position 1053, as a stop codon follows that position. The region of nucleotide position 1057–1229 represents the 3'-untranslated region with a poly(A) tail.

Oligonucleotide primers 11 and 12 (Table I) were synthesized from the data in Fig. 1. They were used to amplify the cDNA encoding full length hyaluronidase, with flanking *Bam*HI and *Bgl*II restriction sites at the 5' and 3' ends, respectively. The full length cDNA was used for expression in bacteria as will be described later.

For sequence analysis, all fragments were cloned into a plasmid vector. The DNA sequence in Fig. 1 was assembled from the data of 5 clones from 3'-RACE, 4 clones from 5'-RACE and 1 clone from specific PCR for expression of hyaluronidase. There are sufficient overlaps of the sequence data of these clones such that every nucleotide position in Fig. 1 represents the consensus of 4 or more clones. The only exception is the region of position 1–45, which was obtained from 2 clones. There are several mutations of these clones which are listed in Table II. Most of them are silent mutations, but 2 of them

result in amino acid substitutions. These mutations may be due to infidelity of base incorporation in PCR, or they may represent allelic forms.

The amino acid sequence from the DNA data in Fig. 1 indicates that hyaluronidase has 331 amino acid residues with a molecular mass of 38,929 daltons, as compared to a molecular mass of about 42 kDa from SDS-gel electrophoretic data. The difference in molecular mass suggests that the natural hyaluronidase is a glycoprotein, as the translated sequence has a potential asparagine glycosylation motif of Asn-X-Thr/Ser at residue 79–81.

Sequence Similarity of Hornet Hyaluronidase Dol m 2 with Other Proteins—A sequence search was made at the National Center for Biotechnology Information using the BLAST network service (25). The search revealed that hornet venom hyaluronidase has 56% sequence identity with honey bee hyaluronidase which contains 349 residues (11). Both venom hyaluronidases show significant sequence homology (25–27%) with a membrane protein of guinea pig, human, monkey, and mouse sperm (12, 13). This membrane protein, known as PH-20, is believed to play a role in sperm-egg adhesion. The human PH-20 was recently found to have hyaluronidase activity (14). The sequence comparisons of bee, hornet, and human proteins are shown in Fig. 2.

Expression of Hornet Hyaluronidase Dol m 2—Attempts to express hornet hyaluronidase in bacteria were made with recombinant pQE8 or 12 vector (QIAGEN). The recombinant plasmids were constructed by ligation of hyaluronidase-encoding cDNA with *Bam*HI and *Hind*III restriction enzyme-cut pQE8 or with *Bam*HI- and *Bgl*II-cut pQE12. The required hyaluronidase-encoding cDNA for pQE12 ligation was obtained from clone 12 in Table III. This clone has 2 mutations at base 199 and 642. Only the mutation at base 199 resulted in a codon change, phenylalanine substitution for isoleucine at residue 47, and it fortuitously eliminated a *Bgl*II site in the coding region of hyaluronidase. The required cDNA for pQE8 ligation was

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Y I Y H H R * H L P P N F S R S N C E K
TATATATATCACCACCGATGACATCTCCCGCCTAACTTTCCAGATCGAATTGCGAAAAA 60
S E R P K R V F N I Y W N V P T F M C H 20
TCCGAGAGACCGAAAAGAGTCTTCAACATTATTGGAACGTTCCCTACCTTTATGTGTCAT 120
O Y G L Y F D E V T N F N I K H N S K D 40
CAGTATGGCCTATACTTCGACGAGGTTACAAATTTTAAATATAAAGCATAATTCTAAGGAC 180
D F O G D K I S I F Y D P G E F P A L L 60
GATTTCAGGGTGACAAGATCTCAATTTTATGATCCTGGAGAATTCCCGGCATTGTTG 240
P L K E G N Y K I R N G G V P Q E G N I 80
CCGCTCAAAGAAGGCAATTATAAGATAAGAAACGGAGGAGTTCCTCAAGAAGGTAACATA 300
T I H L Q R F I E N L D K T Y P N R N F 100
ACGATACATCTCCAAAGATTATTCGAAAAATTTGGATAAAACATATCCAAATAGGAACCTTC 360
N G I G V I D F E R W R P I F R Q N W G 120
AACGGTATCGGTGTGATCGACTTTGAAAGATGGAGACCGATCTTCCGACAAAATTTGGGGC 420
N M M I H K K F S I D L V R N E H P F W 140
AATATGATGATTCATAAGAAGTTTCAATAGACCTAGTTCGCAATGAACATCCATTCTGG 480
D K K M I E L E A S K R F E K Y A R L F 160
GATAAAAAGATGATCGAATTGGAGGCATCTAAGAGGTTTGAAAAATATGCCAGACTTTTC 540
M E E T L K L A K K T R K Q A D W G Y Y 180
ATGGAGGAAACTTTGAAATTGGCCAAAAGACTAGGAAGCAGGCCGATTTGGGGCTATTAC 600
G Y P Y C F N M S P N N L V P D C D A T 200
GGATATCCCTACTGTTTAAATATGTCGCCATAATCTCGTACCCGATTTGTGACGCTACA 660
A M L E N D K M S W L F N N Q N V L L P 220
CGGATGCTCGAGAACGACAAGATGTCGTGGCTGTTCAATAATCAAAATGTACTTCTACCA 720
S V Y I R H E L T P D Q R V G L V Q G R 240
TCCGCTCTATATTAGACACGAACGACCCCTGATCAAGAGTTGGTTTAGTCCAAGGAAGA 780
V K E A V R I S N N L K H S P K V L S Y 260
GTGAAGGAAGCTGTTAGGATATCGAATAATTAAAACATTCACCGAAAGTGCTCTCTTAT 840
W W Y V Y Q D D T N T F L T E T D V K K 280
TGGTGGTACGTGTATCAGGACGATACAAACACTTTTCTTACCGAGACCGACGTGAAAAAG 900
T F Q E I A I N G G D G I I I W G S S S 300
ACTTTCCAAGAGATAGCGATTAAACGGTGGGATGGTATCATTATATGGGGTAGCTCGTCC 960
D V N S L S K C K R L R E Y L L T V L G 320
GACGTAACACAGCTTAAGTAAATGTAAGAGATTACGGGAGTATCTGTTGACGGTTTGGGA 1020
P I T V N V T E T V N * 340
CCAATCACGGTTAACGTGACGGAACCGTCAACTAAAGATTATCCCTAAACTTTTAGTAC 1080
AATCTATGTAACTCTTGCCGATGGCGATAGGTGTGTTCAATGATCTGCTTTGCGAACGC 1140
TATCGATGCTGCAACGATGAATACTGCGACAATGCCATCACATTGAAAAGACTTTTCGCA 1200
GGAAGGAAAAAAAAAAAAAAAAAAAAAAAAA 1229

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FIG. 1. cDNA and amino acid sequences of hornet hyaluronidase (Dol m 2). Nucleotide and amino acid positions are numbered on the right. Numbering of amino acid residues begins and ends at its N and C termini of serine and asparagine, corresponding to nucleotide positions of 61–63 and 1051–1053. The underlined amino acid sequence was established by Edman degradation. The GenBank accession number for the above sequence is L34548.

obtained by PCR amplification of the cDNA in clone 12 with primers 11 and 13 in Table I.

The recombinant pQE8 or 12 plasmid was used to transform competent M15(pREP4) bacteria. On induction with isopropylthiogalactoside, recombinant proteins were expressed by the bacteria and could be isolated by metal ion chelation chromatography because of the presence of a hexahistidine tag. The expected sequence of the recombinant hyaluronidase (hya) from pQE8 is MRGSH₆GS-hya-KLN, and that from pQE12 is MRGS-hya-SRH₆. Their expected molecular masses are 40,539 and 40,340 daltons, respectively. On SDS-gel electrophoresis, the purified proteins from pQE8 contained mainly two bands of about 27 and 12 kDa (results not shown), and the purified proteins from pQE12 also contained two bands of about 40 and 28 kDa (Fig. 3A, lane 2). On immunoblotting, both bands from

pQE12 expression bound hyaluronidase-specific mouse antisera (Fig. 3B, lane 2), but those from pQE8 expression did not (results not shown).

Attempts to separate the 40- and 28-kDa proteins from pQE12 expression by various chromatographic means were unsuccessful. These two proteins were transferred from SDS gel by electroblotting onto Immobilon PSQ membrane, then sequenced by Edman degradation for 20 cycles. The 40-kDa protein contains the expected N-terminal sequence of Dol m 2 preceded by the tetrapeptide of MRGS. The size and the sequence data of the 40-kDa protein, as well as the immunoblot data, all suggest that it is the desired recombinant protein. The recombinant protein is probably acylated at its N terminus, as the yield of amino acid phenylthiohydantoin at each step of degradation is low for the 40-kDa protein when compared to

TABLE II
Sequence mutations of clones from 3'- and 5'-RACE and expression PCR

The consensus sequence is given in Fig. 1. A mutation at position 151 results in a codon change from AAT for asparagine to GAT for aspartic acid, and at position 199 from ATC for isoleucine to TTC for phenylalanine. Mutations at positions 251, 259, and 642 did not result in codon changes. The remaining mutations are in the 3'-untranslated region.

Source/clone	Nucleotides at indicated positions									
	151	199	251	259	642	1064	1137	1154	1172	1184
5'-RACE										
9	A									
19	A	A								
32	A	A								
39	G	A								
Expression										
12	A	T	A	T	C					
3'-RACE										
1	A	A	G	C	A	T	G	G	A	T
2		A	A	T	A	T	A	A	A	T
3						T	A	A	G	T
4		A	A	T	A					
7						C	A	A	A	A
Consensus	A	A	A	T	A	T	A	A	A	T

bee	PDNNKTVREFNVYWNVPTFMCHKYGLRFEEVSEKYGILQNWMDKFRGEEI	50
hornet	SERPKRVFNIYWNVPTFMCHQYGLYFDEVNT-FNIKHNSKDDFQGDKI	47
human	APPVIPNVFPLWAWNAPSEFCLGKFDEPLDMSL-FSFIGSPRINATGQGV	88
bee	AAILYDPGMFPALLKDPN-GNVVARNGGVPQLGNLTKHLQVFRDHLINQIP	99
hornet	SIFYDPGEFPALLPLKE-GNYKIRNGGVPQEGNITIHQRFIENLDKTYP	96
human	TIFYVDRLGYYPYIDSITGVTV--NGGIPQKISLQDHLDAKAKDITFYMP	136
bee	DKSFGVGVIDFESWRPIFRQNWASLQPYKKLSVEVVRREHPFWDQORVE	149
hornet	NRNFNGIGVIDFERWRPIFRQNWGNMMIHKKFSIDLVRNEHPFWDKKMIE	146
human	VDNL-GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQLSLTEAT	185
bee	QEAKRRFEKYQGLFMEETLKAAKRMRPAANWGYYAYPYCYNLTNPQPS--	197
hornet	LEASKRFEKYARLFMEETLKLAKKTRKQADWGYGYGYPCFNMSPNNLV--	194
human	EKAKQEFEEKAGKDFLVETIKLGLLRPNHLWGYYLFPDCYNHHYKPGYN	235
bee	AQCEATTMQENDKMSWLFESQEDVLLPSVYLRWNLTSGERVGL-VGGRVKE	246
hornet	PDCDATAMLENDKMSWLFNNQNVLLPSVYIRHELTPDQORVGL-VQGRVKE	243
human	GSCFNVEIKRNDLSWLWNSTALYPSIYLNTQQSPVAAT-LYVRNRVRE	284
bee	ALRIARQMTTSRKKVLPYYWYK---YQDRRDITDLSRADLEATLRKITDLG	293
hornet	AVRISNNLKHSP-KVLSYWWYV---YQDDTNTFLTETDVKKTFQEIANG	289
human	AIRVSKIPDA--KSPLPVFAYTRIVFTDQVLKFLSQDELVYTFGETVALG	332
bee	ADGFIIWGSSDDINTKAKCLQFREYLNNELGPAVKRIALNNNANDRLTVD	343
hornet	GDGIIIWGSSSDVNSLSKCKRLREYLLTVLGPITVNVTTETVN-----	331
human	ASGIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLAAKMCSQVLCQ	382

FIG. 2. Sequence comparison of honey bee and hornet venom hyaluronidases and human sperm protein PH-20. Alignment starts with residue 1 for both hyaluronidases and residue 40 for PH-20. Bee venom hyaluronidase and PH-20 contain 349 and 495 residues, respectively. Gaps, indicated by *hyphens*, were added to maximize sequence homology. *Filled circles* indicate residues of bee or human protein identical with those of hornet protein.

that for the 28-kDa protein.

The 28-kDa protein contains a sequence corresponding to a fragment beginning at residue 123 of Dol m 2. To test whether the 28-kDa protein was generated by proteolysis of the recombinant Dol m 2, site-directed mutagenesis of the hyaluronidase cDNA was made by a PCR method (21) to replace the adenine base at position 424 with a cytosine. The resulting codon

change yields a mutant with a Leu¹²²-Met¹²³ bond in place of the natural Met¹²²-Met¹²³ bond. Bacteria were transformed with recombinant pQE8 containing the mutant Dol m 2, and the desired mutant protein was expressed on induction with isopropylthiogalactoside. After purification by successive metal ion chelation and reverse phase chromatography, the mutant protein was isolated in a yield of about 2.5 mg/liter of culture.

TABLE III

White face hornet (*Dolichovespula maculata*) and honey bee (*Apis mellifera*) venom allergens and their sequence identity with other proteins

Allergens	Other proteins with partial sequence identity	Refs.
Honey bee		
Api m 1; phospholipase A ₂	Mammalian pancreatic phospholipases	26, 27
Api m 2; hyaluronidase	Mammalian testis protein PH-20	11
Api m 3; melittin ^a	Calmodulin binding protein	28
	Mammalian protein with phospholipase A ₂ stimulating activity	29
	Human complement C9	30
White face hornet		
Dol m 1; phospholipase A ₁	Mammalian lipases	7
Dol m 2; hyaluronidase	Mammalian testis protein PH-20	Present work
Dol m 5; antigen 5	Mammalian testis protein Tpx-1 plant pathogenesis-related proteins	6, 31

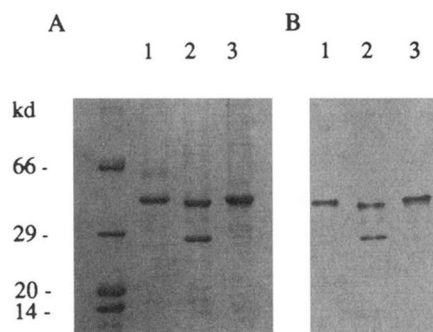
^a Sequence identity for melittin and other proteins was inferred from cross-reaction by antibody assays.

FIG. 3. SDS-gel electrophoresis and immunoblot of natural and recombinant hornet hyaluronidase Dol m 2 in patterns A and B, respectively. Lane 1, natural protein; lane 2, recombinant protein with Ile to Phe mutation at residue 47 from pQE12 vector; and lane 3, recombinant protein with Met to Leu mutation at residue 122 from pQE8 vector.

The purified mutant protein gave mainly one band of about 40 kDa on SDS-gel electrophoresis and immunoblotting (Fig. 3, A and B, lane 3). The immunoblot was detected with mouse sera specific for natural and recombinant Dol m 2 with identical results, but only the results with recombinant Dol m 2 specific sera are shown in the figure.

Hyaluronidase Activity of Recombinant Dol m 2—The recombinant protein with Ile to Phe or Met to Leu mutation at residues 47 and 122, respectively, was found to be devoid of enzymatic activity as measured by the depolymerization of hyaluronic acid (23). This is not surprising since the recombinant proteins lack the disulfide bonds of the natural protein. Unsuccessful attempts were made to refold the recombinant proteins by air oxidation of its cysteinyl residues in pH 7.4 buffer with or without 1 or 2 M guanidine hydrochloride. These attempts were also made in the presence of 2.5 mM 2-mercaptoethanol to promote exchange of any incorrectly paired disulfide bonds. In no case did we obtain >0.03% regeneration of the activity of the natural protein.

Immunological Properties of Recombinant Dol m 2—Although mouse antisera specific for natural Dol m 2 and recombinant Dol m 2 with Ile to Phe mutation at residue 47 appear to behave similarly on immunoblots (Fig. 3), they differ in their titers on enzyme-linked solid-phase immunoassay. One antiserum sample specific for natural Dol m 2 showed a titer about 100-fold higher on solid-phase natural Dol m 2 than on recombinant Dol m 2 with an Ile to Phe or Met to Leu mutation. But an antiserum sample specific for the recombinant Dol m 2 showed the same titer on solid-phase recombinant Dol m 2 as on natural Dol m 2. These results suggest that the recombinant and the natural proteins have common B cell epitopes of the continuous type, and that the recombinant protein does not have the discontinuous type B cell epitopes of the natural protein.

Data on antigen-stimulated proliferation of spleen cells from mice immunized with natural Dol m 2 or recombinant Dol m 2

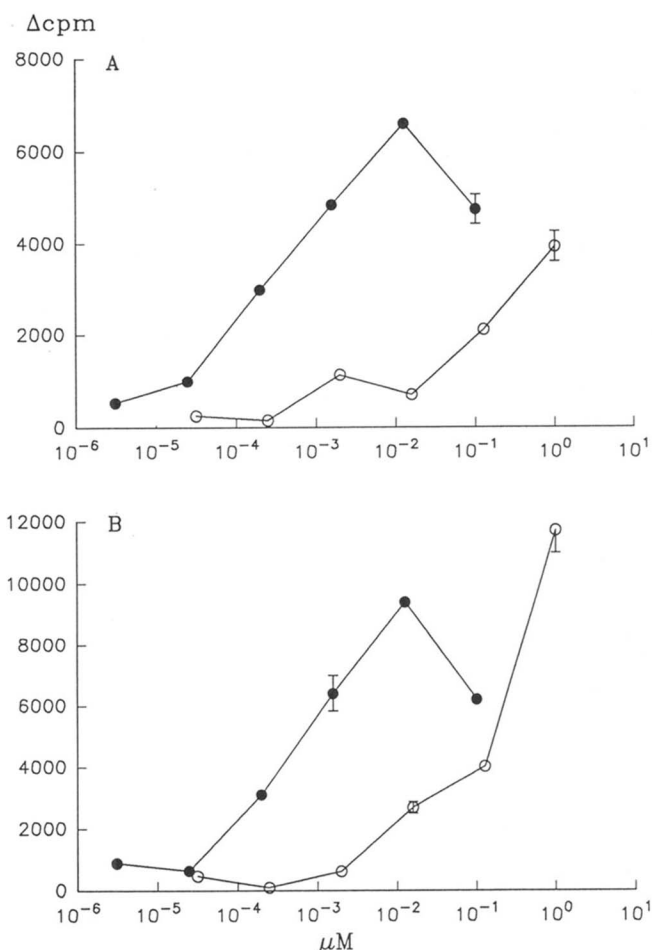


FIG. 4. Proliferation assay with spleen cells from mice after 5 immunizations with natural hornet hyaluronidase and recombinant hornet hyaluronidase with Ile to Phe mutation at residue 47, in patterns A and B respectively. The stimulating antigens are the natural hornet protein (filled circles) and the recombinant protein with Ile to Phe mutations at residue 47 (open circles). Cell proliferation was measured by the uptake of [³H]thymidine. Background proliferation was 3400 and 6000 cpm for patterns A and B, respectively. Error bars represent S.D. of triplicate measurements.

with an Ile to Phe mutation are given in Fig. 4, A or B, respectively. In both graphs, lower concentrations of the natural protein were required for maximal proliferation than for the recombinant protein, and both graphs suggest similar maximal stimulations for both proteins. The lower concentration of the natural protein required for maximal stimulation may reflect its ease of uptake and/or processing by the appropriate cells for presentation to T cells. These data suggest that the recombinant and natural proteins have common T cell epitopes.

Antigen Cross-reactivity of Hornet and Bee Venom Hyalu-

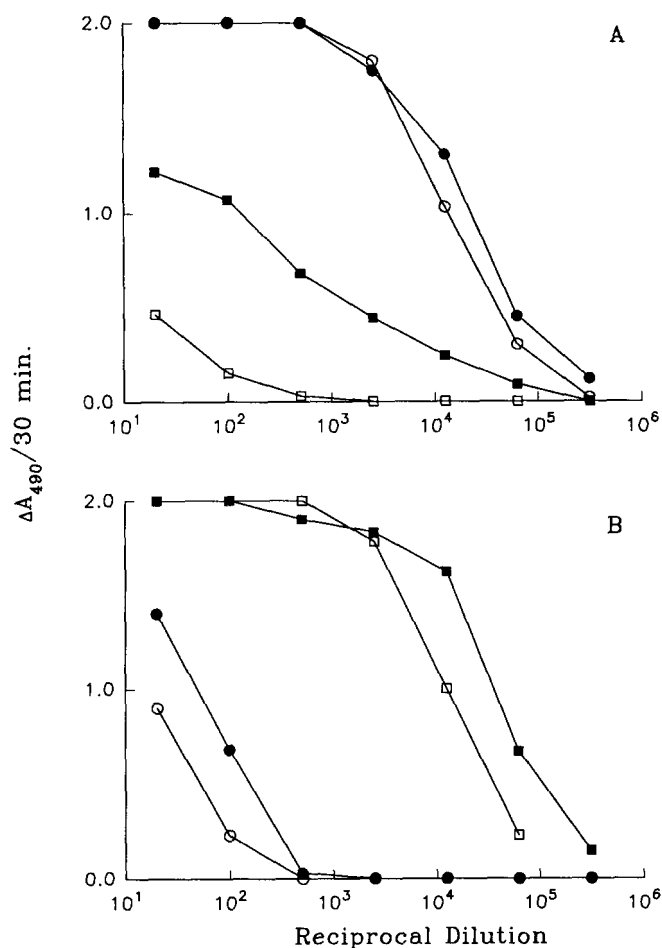


FIG. 5. Enzyme immunoassay of hornet and bee venom hyaluronidase-specific mouse sera on solid-phase hornet and bee hyaluronidases (patterns A and B, respectively). Hornet hyaluronidase-specific sera of week 5 and 7 bleedings are designated by open and closed circles, and those for bee-specific sera are designated by open and closed squares, respectively.

ronidases—Sera from BALB/c mice which were immunized with hornet or bee hyaluronidase showed high antibody titer for the immunogen, but a weak cross-reaction of hornet and bee hyaluronidases was detected by direct enzyme immunoassay as shown in Fig. 5, A and B. A higher degree of cross-reaction was observed with the hornet specific sera than with the bee specific sera. The degree of cross-reaction increased with the number of immunizations, as shown by the results for sera from week 5 and 7 bleedings.

To study cross-reaction at the T cell level, proliferation assays with spleen cells from hornet or bee hyaluronidase immunized mice were carried out. Spleen cells from mice after 2 immunizations with hornet hyaluronidase at week 3 responded equally well on stimulation with hornet or bee hyaluronidase (Fig. 6A), and the magnitude of spleen cell response to hornet protein increased after 3 more immunizations with hornet protein at week 9, but the magnitude of response to bee protein remained about the same or decreased slightly (Fig. 6B). Similar studies showed that the spleen cells from mice after 2 immunizations with bee hyaluronidase at week 3 responded strongly on stimulation with bee protein and weakly with hornet protein (Fig. 6C); this difference persisted after 3 more immunizations at week 9 (Fig. 6D).

DISCUSSION

In Table III are listed the known allergens of honey bee and white face hornet venoms, of which amino acid sequences have

been determined by chemical sequencing or by molecular cloning. Bee venom has three allergens: hyaluronidase, phospholipase A₂, and melittin, which is a 26-residue peptide. Hornet venom also has three allergens: antigen 5, hyaluronidase, and phospholipase A₁. The other vespids, yellow jackets and wasps, have allergens homologous to those of hornets.

As shown in Fig. 2, there is 56% sequence identity of hornet and bee hyaluronidases. The sequence identity of hornet and bee hyaluronidases raises the possibility that they may have common or cross-reacting B and T cell epitopes. This is supported by the immunoassay data with antibodies from mice immunized with hornet or bee hyaluronidase (Fig. 5) and by the proliferative assay with spleen cells from these mice (Fig. 6). The antigenic cross-reactivity of yellow jacket and bee hyaluronidases has been detected by RAST inhibition assay with sera from allergic patients (32, 33). These findings together may explain the common observation that the majority of insect allergic patients exhibit multiple sensitivity to bees and vespids (1, 2). Thus, the observed multiple sensitivity can be a consequence of multiple exposures to different insects and/or the cross-reactivity of bee and vespid hyaluronidases.

In addition to the insect venom allergens, the amino acid sequences of 70 or more allergens from pollen, mites, animal danders, molds, foods, etc. are known (*cf.* Ref. 15). These allergens have different sequences and biological functions. One common feature of these allergens is their varying extents of sequence identity with other proteins in our environment. This is exemplified by the data in Table III for insect venom allergens. Each venom allergen in Table III has at least 20% sequence identity with a mammalian protein. In each case there is more than one region of sequence identity ranging in size from penta- to longer length peptides. These regions of sequence identity can function as common B or T cell epitopes, since the minimal sizes of B and T cell epitopes are in the range of penta- to decapeptides.

Normally, a host does not mount immune responses to self-proteins. It is possible that insect allergic patients recognize their self proteins, so that they are primed to respond to the cross-reacting venom allergens. This would explain why susceptible people can become sensitized readily on exposure to microgram amounts of allergen. This hypothesis remains to be proven.

It has been reported that in people with a high total IgE level, positive skin venom tests were more frequent in males than in females (34). Two of the venom allergens in Table III have sequence identity with mammalian testis proteins. Vespid antigen 5s have sequence identity with a mammalian testis protein, and hornet and bee hyaluronidases have sequence identity with another testis protein. When hornet antigen 5 was tested in male and female mice of BALB/c strain, no difference in immunogenicity for IgG antibody or for T cell response was observed in male or female animals.²

One problem in immunotherapy of patients is the limited dose of allergens which can be administered safely (1, 2, 4). Recent model experiments showed that treatment of mice with the dominant T cell epitopes of the major cat or mite allergen (35, 36) induced suppression of allergen-specific immune response. Although the exact mechanism of the reported immunosuppression is still under study (37, 38), these findings are of interest. Peptides are less likely to induce systemic reactions in patients than multivalent allergens with their full complement of B cell epitopes of the continuous and discontinuous types.

The present work on recombinant hornet hyaluronidase, as well as our published report (39) on recombinant hornet anti-

² T. P. King, unpublished results.

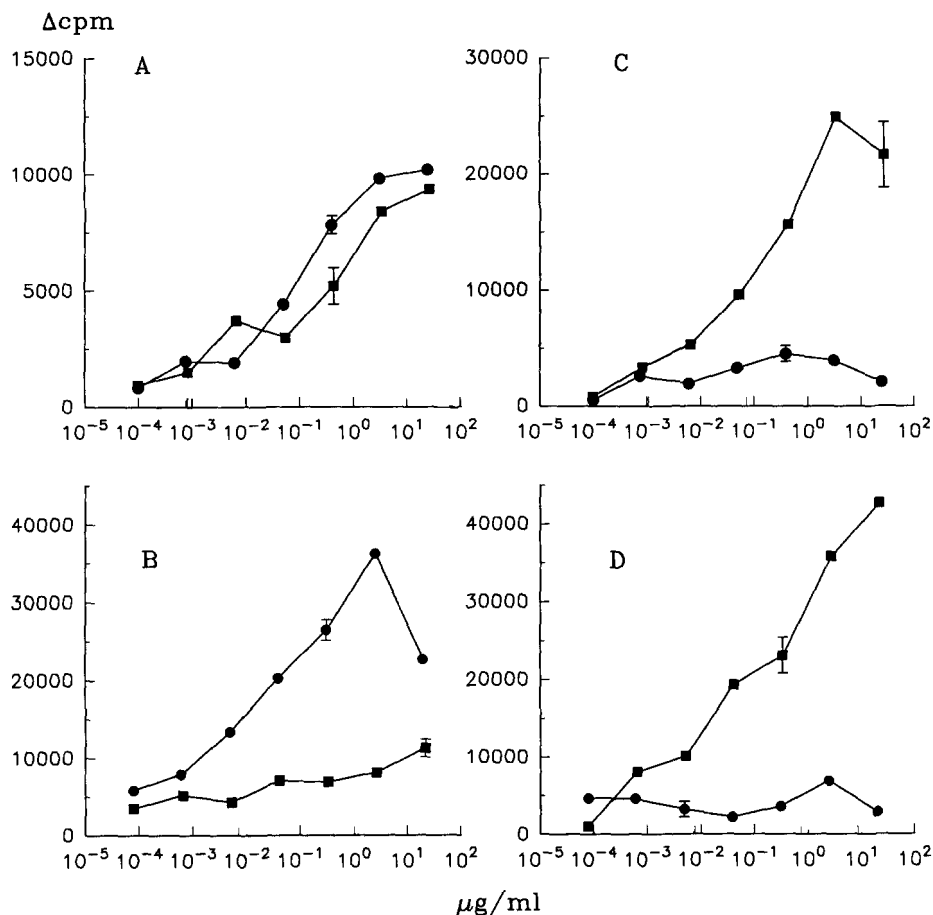


FIG. 6. Proliferation assay with spleen cells from mice after 2 or 4 immunizations with hornet venom hyaluronidase (patterns A and B, respectively) and from mice after 2 and 4 immunizations with bee venom hyaluronidase (patterns C and D, respectively). The stimulating antigens are hornet hyaluronidase (filled circles) and bee hyaluronidase (filled squares). Cell proliferation was measured by the uptake of [³H]thymidine. Background proliferation was 8000 ± 2000 cpm. Error bars represent S.D. of triplicate measurements.

gen 5 and our unpublished results on recombinant hornet phospholipase A₁, is relevant to studies on the possible use of peptide fragments as immunotherapeutic reagents. All three recombinant proteins lack the native conformation of the natural proteins, as indicated by the lack of enzymatic activity where applicable, and by the absence of the discontinuous type B cell epitopes of the natural proteins. Nonetheless, they and their fragments retain the T cell epitopes of the natural proteins, as T cell epitopes consist only of sequentially adjacent amino acid residues (40).

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