

IMMUNOCHEMICAL OBSERVATIONS OF ANTIGEN 5, A MAJOR VENOM ALLERGEN OF HORNETS, YELLOWJACKETS AND WASPS

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Abstract—Antigen 5 is a venom protein of hornets, yellowjackets and wasps, and it is an important allergen for insect-sensitive patients. Antigen 5s from these insects are found to have similar amino-terminal sequences for their first 20 residues, and to have similar cyanogen bromide cleavage patterns. A partial sequence homology of antigen 5s and scorpion neurotoxins is observed.

Antigen 5s from these insects show varying extents of cross reactivity when tested with mouse polyclonal antibodies by enzyme immunoassay and they show limited or no cross reactivity with mouse monoclonal antibodies. Chemically modified antigen 5s shown greatly decreased binding of antigen 5-specific mouse polyclonal antibodies. The modified antigen 5s include its reduced and carboxy-methylated derivatives and its cyanogen bromide fragments.

INTRODUCTION

Hornets, yellowjackets and wasps belong to the group of insects known as vespids. Their stings can cause allergy in sensitive people. Three of their venom proteins are known to be allergens. They are hyaluronidase, phospholipase A₁ and antigen 5 of as yet unknown biochemical function, and they have mol. wts of about 44,000, 36,000 and 22,000 respectively. The homologous proteins of these vespids have similar biochemical properties which include their mol. size and charge, their amino acid composition and where applicable, their enzymatic property (King *et al.*, 1978, 1983, 1984, 1985).

Vespid allergic people usually have multiple sensitivity to venoms of hornets, yellowjackets and wasps (Lichtenstein *et al.*, 1979; Reisman, 1985). This multiple sensitivity can be a consequence of multiple exposure to different vespids and/or immunologic cross reactivity of the homologous venom proteins. Immunoassays with specific mouse antisera have demonstrated that the homologous antigen 5s and hyaluronidases of vespids show varying extents of antigenic cross reactivity while the phospholipases show no detectable cross reactivity (King *et al.*, 1985).

To learn more about vespid antigen 5s, further biochemical and immunochemical studies were made. These studies were carried out with antigen 5s from white-face hornet (*Dolichovespula maculata*), yellow hornet (*Dolichovespula arenaria*), yellowjacket (*Vespa vulgaris*) and wasp (*Polistes annularis*). Two species of hornets were chosen as their venom proteins are known to be antigenically dissimilar (King *et al.*, 1978). Most known species of yellowjackets,

with the exception of *V. squamosa* (King *et al.*, 1983, 1987; Hoffman, 1985), have antigenically similar venom proteins, and this is also the case with most species of wasps (King *et al.*, 1984).

According to a recently proposed system of allergen nomenclature (Marsh *et al.*, 1986), each purified allergen will be designated by a combination of four alphabets and one Roman numeral; the first three and the fourth alphabets indicate the genus and the species of the allergen source respectively, and the Roman numeral indicates the component in the order of its isolation. Thus the antigen 5s given in the preceding paragraph are designated as Dol m V, Dol a V, Ves v V, Pol a V and Ves s V, respectively. We will use the common names of these insects in this paper as most readers are familiar with the common names of insects but not with the exact scientific names in the allergen nomenclature.

MATERIALS AND METHODS

Antigen 5s were isolated from venom sac extracts of white-face hornet (*D. maculata*), yellow hornet (*D. arenaria*), yellowjacket (*V. vulgaris*) and wasp (*P. annularis*). The venom sac extracts were products of Vespa Laboratories, Spring Mills, PA. The isolation procedures for antigen 5s of yellowjackets and wasps were as reported earlier (King *et al.*, 1983, 1984).

For white-face hornet venom, a modified procedure (King *et al.*, 1978) was used. The venom sac extract (about 100 mg protein as measured by the manufacturer) was dissolved in 6 ml of 0.05 M NH₄Ac buffer (pH 4.75) containing 5 mM phenylmethanesulfonyl fluoride. After 1 hr at 20°C, it was dialyzed against the same buffer then applied to a

column (5×0.9 cm) of heparin-Sepharose (Pharmacia). The column was eluted with a linear gradient of 4.17 mM NaCl/ml in 0.05 M NH_4Ac buffer (pH 4.75) and the eluant was analyzed by absorbance at 230 nm. The large broad peak eluted at 0.06–0.26 M NaCl contained antigen 5 and the small broad peak at 0.3–0.4 M NaCl contained hyaluronidase and phospholipase A_1 (King *et al.*, 1978, 1984). The broad peak was applied directly to an SE-53 cellulose column (20×0.9 cm) which was eluted at 60 ml/hr with a linear gradient of 1.67 mM NaCl/ml in 0.05 M NH_4Ac buffer (pH 4.75). Three isoelectric forms 1, 2 and 3 of antigen 5 were obtained at about 0.32, 0.38 and 0.41 M NaCl respectively. Their yields were 0.27, 0.50 and 0.33 mg/100 mg of venom sac extract, respectively: a value of $E_{280}^{1\%} = 18$ was used to calculate the concn of antigen 5 (King *et al.*, 1978). Form 3 gave a single band of about 22,000 mol. wt on sodium dodecylsulfate gel electrophoresis (Laemmli, 1970) while forms 1 and 2 were contaminated with components of mol. wt of about 40,000 and 10,000. The contaminants were removed by gel filtration on Sephadex G-100 (King *et al.*, 1978) for samples used in sequence analysis. These isoelectric forms were shown previously to be immunochemically indistinguishable by immunodiffusion and by amino acid analysis.

For yellow hornet venom, the procedure was similar to that given above for white-face hornet. The venom was separated on a heparin-Sepharose column. The first broad peak which was eluted with the NaCl gradient contained antigen 5 and phospholipase and the second peak contained hyaluronidase. The first peak was conc. and dialyzed against 0.05 M Tris-HCl buffer of pH 7.95 for affinity chromatography on Sepharose containing bound 1-(8-carboxyoctyl)-2-hexadecyl-rac-glycero-3-phosphorylcholine (See King *et al.*, 1984). The unbound peak contained antigen 5 which was conc. and dialyzed for chromatography on SE-53 cellulose as given above for white-face hornet antigen 5. Two isoelectric forms 1 and 2 of yellow hornet antigen 5 were eluted at about 0.39 and 0.42 M NaCl and their yields were 0.76 and 0.52 mg/100 mg of venom sac extract, respectively. On sodium dodecylsulfate gel electrophoresis, form 2 gave a single band of mol. wt of about 22,000 while form 1 showed a trace contaminant of about 24,000.

Reduced and S-carboxymethylated antigen 5. Dithiothreitol (30 mM) reduction of protein (about 0.5 mg, 23 nmole) was made in 1 ml of 8 M guanidine hydrochloride containing 0.1 M Tris-HCl buffer of pH 8.6 for 5 hr at 25°C. S-carboxymethylation of the reduced protein was carried out after adding iodoacetic acid (12 mg, 65 μ mole) and 2 M Tris (25 μ l, 50 μ mole). After 1 hr or more, the reaction mixture was applied to a 4×0.9 cm column of Polyosil reversed phase C18 silica gel (25–40 μ m, Rainin) followed with a 10 ml wash of 0.1% trifluoroacetic acid. The column was eluted with a linear i-propanol

gradient (0.75%/ml) at 60 ml/hr. The modified protein, eluted at about 30% i-propanol concn, was detected by absorbance at 220 nm. Its yield of 50–70% was estimated by absorbance at 280 nm following dissolution of the lyophilized protein in 0.02 N acetic acid. On sodium dodecylsulfate gel electrophoresis the modified protein migrated as a band of about 28,000 mol. wt. This is in contrast to the native protein which migrated as a band of about 22,000 mol. wt.

Cyanogen bromide cleavage of antigen 5. This was carried out with about 1 mg of protein in 0.2 ml of 3.5 M cyanogen bromide in 75% formic acid. After being kept overnight at 25°C, the reaction mixture was lyophilized. The cleavage mixture was analyzed by sodium dodecylsulfate gel electrophoresis with or without mercaptoethanol reduction or by gel filtration on a 200×0.9 cm column of Sephadex G-50 which was eluted with 1 N acetic acid at 12 ml/hr.

Edman degradation. This was carried out with the reduced and carboxymethylated antigen 5 (0.2–0.5 nmole) on an Applied Biosystems Model 470A gas phase sequencer. The amount of protein sequenced was determined by amino acid analysis of a separate aliquot of the sample. Analyses of phenylthiohydantoin amino acids were made with an Applied Biosystems 120A PTH analyzer or a Hewlett-Packard 1084A liquid chromatography system with a Dupont Zorbax ODS column. The initial yields of phenylthiohydantoin amino acids from these degradations were in the range of 80–95% and their repetitive yields were in range of 86–93%.

Carboxypeptidase digestions. About 0.5 nmole of the reduced and carboxymethylated antigen 5 was digested with 5 μ g of carboxypeptidase A, B or Y (Sigma Biochemical) in 20 μ l of 0.02 N sodium phosphate buffer of pH 7.4. After 1 hr at 25°C the digest was diluted with 100 μ l of 0.2 N sodium citrate buffer of pH 2.2 and used directly for amino acid analysis.

Immunization of mice. Groups of BALB/c mice were immunized by one of the following two methods. With method A, mice were immunized i.p. each with 0.2 ml of a mixture of 10 μ g/ml antigen 5 and 5 mg/ml alum (Roveltella and Ovary, 1969) in 0.05 M sodium phosphate buffer of pH 6.4 at weeks 0, 3, 5 and 11. Their sera were collected at weeks 3, 5, 7 and 13. With method B, mice were immunized i.p. each with 0.2 ml of an emulsion of nine parts of complete Freund's adjuvant (CalBiochem) and one part of 0.06–0.20 mg/ml of antigen in 0.02 M NH_4Ac buffer of pH 4.75 at weeks 0, 3, 4, 5 and 8 (King *et al.*, 1985; Tung *et al.*, 1976). Immune ascites were collected beginning on week 5 but only the ascites collected during weeks 13 and 14 were pooled and used in this study.

Isoelectric forms 3 and 2 of white-face and yellow hornet antigen 5s, respectively, were used for immunization of mice.

White-face hornet antigen 5-specific mouse polyclonal antibodies. They were purified by affinity

chromatography of 0.8 ml of immune ascites on an immunosorbent which was prepared by coupling 0.9 mg of white-face hornet antigen 5 to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia) in 5 ml of 0.1 M NaHCO_3 + 0.5 M NaCl at 25°C overnight as directed by the manufacturer. After applying the ascites to the immunosorbent, the column was washed with 0.05 M Tris-HCl buffer (pH 7.95) + 0.10 M NaCl to remove non-specific proteins. Then the bound antibodies (about 1 mg) were eluted with 0.05 M glycine-HCl buffer (pH 2.0) and the acidic eluates were immediately neutralized with Tris-HCl buffer.

Antigen 5-specific mouse monoclonal antibodies. Hybridomas were obtained by fusion of antigen 5-stimulated spleen cells with plasmacytoma sp 2/0 cells (Kennett, 1980). The spleen cells were taken from mice which were immunized by method A described earlier. The hybrid cells were cloned by limiting dilution to 0.3 cell per well. After cloning, the cells were grown to maximal cell density in the standard hybridoma medium containing 11% horse serum and the resulting culture fluids were conc. 10-fold by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 40% saturation. After dialysis against 0.05 M Tris-HCl buffer of pH 7.95, the concentrates containing the monoclonal antibodies were used directly without further purification. The monoclonal antibodies studied were of IgG₁ isotype with kappa light chain as determined with rabbit antibodies from a commercial mouse Ig subtype identification kit (Boehringer-Mannheim).

Solid-phase enzyme immunoassay. This was carried out according to published procedures with minor modifications (Engvall and Perlman, 1972; Giallongo *et al.*, 1982). Solid-phase antigen was prepared by treating wells of flexible polyvinyl plate (Falcon No. 3911) each with 30 μ l solutions of 5 μ g/ml native protein, or 10 μ g/ml modified protein in 0.05 M Tris-HCl buffer of pH 7.95. After overnight at 25°C the wells were filled with diluent buffer which contained 0.1 mg/ml bovine plasma albumin, 0.5 mg/ml Tween-20 and 0.5 M NaCl in 0.05 M Tris-HCl buffer (pH 7.95). After 0.5 hr the wells were washed with more diluent buffer then filled with 20 μ l aliquots of mouse antibody dilutions in diluent buffer. After 1 hr the wells were washed and filled with 20 μ l aliquots

of rabbit anti-mouse IgG (10 μ g/ml protein: Sigma Biochemical). After another hour, the wells were washed and filled with 20 μ l aliquots of 2 μ g/ml horseradish peroxidase conjugate of sheep anti-rabbit IgG (King *et al.*, 1978). After another hour the wells were washed and filled with 50 μ l aliquots of substrate solution for peroxidase (Giallongo *et al.*, 1982). After 0.5 hr the developed color was measured at 490 nm with an EIA reader (Bio-Tek Instrument).

RESULTS

Biochemical studies of vespid antigen 5s. In Fig. 1 are given the amino-terminal sequences of white-face hornet, yellow hornet, yellowjacket and wasp antigen 5s. These sequences were determined by Edman degradation with 5–12 μ g of the reduced and carboxymethylated proteins using the gas phase sequencing technique. To obtain maximal alignment of the amino acid residues of these antigen 5s, gaps were placed in the sequences of hornet and wasp antigen 5s as shown in Fig. 1.

White-face and yellow hornet antigen 5s are both isolated in multiple isoelectric forms from their venoms. Sequence analyses of forms 2 and 3 of white-face hornet antigen 5 gave identical results. For yellow hornet antigen 5 only form 2 of its two isoelectric forms was analyzed.

A computer search of the sequences of known proteins in the Genbank and the Dayhoff databases revealed a partial homology of the amino-terminal 20-residue sequences of vespid antigen 5s with the internal sequences of several scorpion neurotoxins. This is shown in Fig. 2.

Carboxypeptidase A digestion of white-face hornet antigen 5 gave no release of amino acids. Carboxypeptidase Y digestion (Hayashi, 1977) released traces of lysine and glycine, while carboxypeptidase B digestion released nearly 0.6 residue each of lysine and glycine together with a lesser amount of arginine. Carboxypeptidase B digestion of yellow hornet, yellowjacket and wasp antigen 5s also released lysine and glycine. Based on the known specificities of carboxypeptidases A, B and Y, these results suggest that the vespid antigen 5s have a common carboxyl

	5	10	15	20	~190
YJ	N N Y C K I K C L K G V H T A C K Y G	//	G	K	
YH	- - - - - () - P - - () T - - L - - - -	//	-	-	
WFH	- - - - - S R - () I - - L - - F -	//	-	-	
W	V D - - - - - P S - () I X - V - Q - -	//	-	-	

Fig. 1. Partial amino acid sequence data of antigen 5s of yellowjacket (YJ), yellow hornet (YH), white-face hornet (WFH) and wasp (W). Amino acid residues which are common to those of YJ antigen 5 are shown as hyphens. Open parentheses represent gaps introduced for maximal alignment of residues of antigen 5s. The symbol // represents intervening peptide sequence between the amino and the carboxyl-terminal regions. The carboxyl-terminal residue number is tentative.

	1									10										20
Yellowjacket	N	N	Y	C	K	I	K	C	L	K	G	G	V	H	T	A	C	K	Y	G
	9																			28
Mexican Scorpion	-	T	G	-	-	Y	E	-	-	-	L	-	D	N	D	Y	-	L	R	E
	19																			38
European Scorpion	-	A	-	-	D	E	E	-	K	-	-	A	E	S	G	K	-	W	-	A
	9																			28
Bark Scorpion	S	D	G	-	-	Y	G	-	-	-	L	-	E	N	E	G	-	D	T	E
	9																			27
Brazilian Scorpion	Y	D	N	-	A	Y	I	-	W	N	Y	D	N	A	Y	(-)	-	D	K	L

Fig. 2. Comparison of partial sequences of yellowjacket antigen 5 and scorpion neurotoxins. The sequence data for neurotoxins of Mexican (*Centruroides noxius*), European (*Buthus europaeus*), bark (*Centruroides sculpturatus*) and Brazilian (*Tytilus serrulatus*) are taken from references Martin *et al.* (1982), Grishin *et al.* (1979), Fontecilla-Camps *et al.* (1982) and Possani *et al.* (1982), respectively. Amino acid residues which are common to those of YJ antigen 5 are shown as hyphens.

terminal sequence of glycyl lysine as indicated in Fig. 1.

In Fig. 3 are shown the sodium dodecylsulfate gel electrophoretic patterns of vespid antigen 5s after CNBr cleavage. Before cleavage, the vespid antigen 5s had nearly identical sizes of about 22,000 mol. wt (results not shown). After cleavage, each antigen 5 gave several prominent bands of mol. wt 14,000–22,000 together with a diffuse band of 5000–10,000 mol. wt. Antigen 5s of white-face and yellow hornets and yellowjacket showed two common prominent bands of 14,000 and 17,000 mol. wt while wasp antigen 5 showed two prominent bands of 17,000 and 22,000 mol. wt. The 22,000 band, which is present in greater amount in CNBr-cleaved wasp antigen 5 than it is in other vespid antigen 5s, nearly disappeared following reduction with mercaptoethanol (lanes 7–10 of Fig. 3). These results suggest the 22,000 band to be a mixture of non-covalently associated aggregates of CNBr cleavage products and incompletely cleaved antigen 5.

After CNBr cleavage followed with mercaptoethanol reduction, the four vespid antigen 5s

each gave several diffuse bands of mol. wt 5000–10,000. Four CNBr peptides expected as vespid antigen 5s were found to contain about three methionine residues per molecule by amino acid analysis (King *et al.*, 1978, 1983, 1984). However it is difficult to conclude from Fig. 3, the number of CNBr peptides generated and their mol. sizes. This is because of the poor gel electrophoretic resolution of CNBr peptides and the probable presence of incompletely cleaved overlapping CNBr peptides. The poor electrophoretic resolution of peptides of mol. wt less than 10,000 was also observed with the reduced and CNBr-cleaved bee venom phospholipase A₂ which is a well-characterized protein of 128 amino acid residues (Shipolini *et al.*, 1974); these results are not shown. Therefore the diffuse CNBr peptide bands in Fig. 3 do not indicate heterogeneity of the vespid antigen 5 samples.

In Fig. 4 is shown the gel filtration pattern of white-face hornet antigen 5 following CNBr cleavage. Two peaks at 55 and 90 ml corresponding to one large and one small fragment were obtained, and on gel electrophoresis they corresponded to the bands of

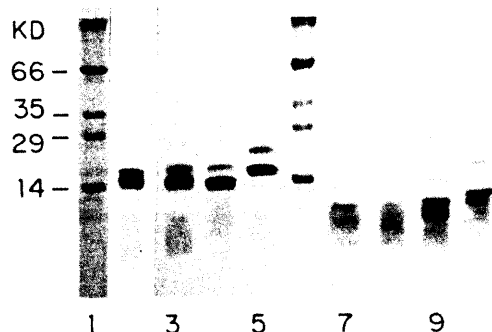


Fig. 3. Sodium dodecylsulfate gel electrophoresis of CNBr-cleaved vespid antigen 5s. Lanes 2–5 contain white-face hornet (form 3), yellow hornet (form 2), yellowjacket and wasp antigen 5s, respectively. Lanes 7–10 contain the same samples of lanes 2–5 but they were reduced with mercaptoethanol. Lanes 1 and 6 contain standard proteins. About a 4 µg sample was applied to each lane of a 12–17% gradient gel.

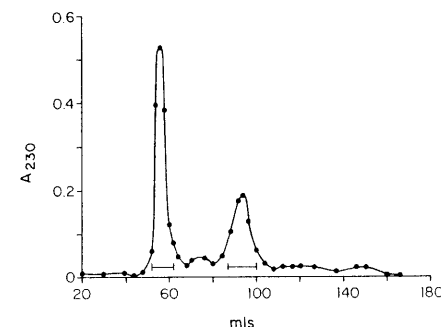


Fig. 4. Gel filtration of CNBr-cleaved white-face hornet antigen 5 on Sephadex G-50. About 0.9 mg of antigen 5 was used. Insulin and glucagon of 5700 and 3500 mol. wt were eluted at 86 and 106 ml, respectively.

14,000–17,000 and 5000–10,000 mol. wt shown in Fig. 3. The small fragment contains the carboxyl-terminal region of antigen 5, as carboxypeptidase B digestion of this fragment gave the same pattern of amino acid release as that of the intact protein.

Antigenic cross reactivity of vespid antigen 5s. This was studied by enzyme immunoassay using polyclonal mouse antibodies on solid-phase autologous or homologous antigen 5s. In Fig. 5(A), (B) and (C) are shown the results of mouse anti-sera specific for white-face hornet, wasp and yellowjacket antigen 5s respectively. All three sera showed binding to autologous and homologous antigen 5s, but they differed in their titers for one-third maximal binding. If one arbitrarily assumes that a 10-fold difference in titers for binding to autologous and homologous antigen 5s as representing significant cross reaction, one will conclude that there is cross reaction of white-face hornet and yellow hornet antigen 5s [Fig. 5(A)], of white-face hornet and wasp antigen 5s [Fig. 5(A) and 5(B)] and of yellowjacket and yellow hornet antigen 5s [Fig. 5(C)].

The results in Fig. 5 were obtained with sera which were collected from mice (group A) at week 13 after four immunizations with antigen 5 in the presence of

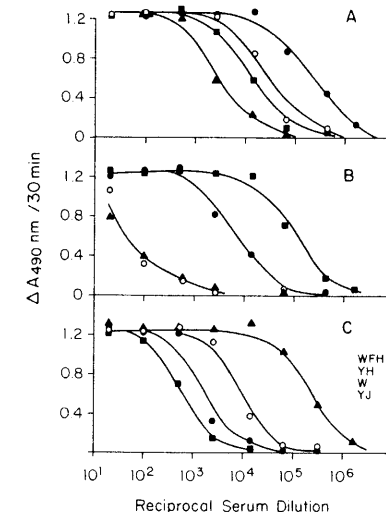


Fig. 5. Enzyme immunoassay of polyclonal mouse antibodies on solid-phase autologous or homologous antigen 5. Antibodies specific for white-face hornet, wasp and yellowjacket antigen 5s were tested in patterns (A), (B) and (C), respectively. The solid-phase antigen 5s were white-face hornet (●), yellow hornet (○), wasp (■) and yellowjacket (▲).

alum as adjuvant, and nearly the same results were obtained with sera which were collected at week 5 after two immunizations (data not shown). Tests were also made with antibodies from mice (group B) immunized with antigen 5 in the presence of complete Freund's adjuvant, and the results with antibodies of group B mice show a similar, but greater, degree of cross reactivity than those of group A do (data not shown).

Enzymes immunoassays were also made with monoclonal antibodies on solid-phase autologous or homologous antigens 5s. The antibody titers for one-third maximal binding of antigen 5s are summarized in Table 1. Two monoclonal antibodies

Table 1. Cross reactions of vespid antigen 5-specific monoclonal antibodies

Monoclonal Abs specific for	Reciprocal titer for 1/3 maximal binding of solid-phase Ag ^{5s}			
	White-face hornet	Yellow hornet	Yellow-jacket	Wasp
A. WFH-Ag5	7 × 10 ²	7 × 10 ²	0	0
B. WFH-Ag5	2 × 10 ⁴	2 × 10 ⁴	0	0
C. YH-Ag5	1	9 × 10 ³	1	1
D. YH-Ag5	8 × 10 ³	1 × 10 ⁴	1	<1
E. YH-Ag5	1	1 × 10 ⁴	1	<1
F. YH-Ag5	<1	1 × 10 ⁴	<1	<1
G. YJ-Ag5	1	1	6 × 10 ³	<1
H. YJ-Ag5	1	1	4 × 10 ³	<1
I. W-Ag5	5	9	7	1 × 10 ¹
J. W-Ag5	3	5	8	3 × 10 ³

*The data were obtained by enzyme immunoassay in the same manner as shown in Fig. 5 for polyclonal antibodies.

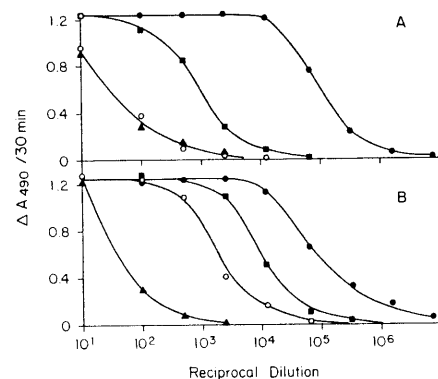


Fig. 6. Enzyme immunoassay of polyclonal mouse antibodies on solid-phase native or modified white-face hornet antigen 5. The antibodies in patterns (A) and (B) were from mice immunized with antigen 5 in the presence of alum or complete Freund's adjuvant (Groups A and B, respectively). The solid-phase antigens tested were native antigen 5 (●), its reduced and carboxymethylated derivative (○), and its CNBr large (■) and small (▲) fragments. Affinity purified antibodies from group B mice were also tested and the results are identical to those of unfractionated antibodies in pattern (B); the concn of the purified antibody solution was 2.5 mg/ml.

specific for each of white-face hornet, yellowjacket and wasp antigen 5s and four monoclonal antibodies specific for yellow hornet antigen 5 were tested. The two monoclonal antibodies specific for white-face hornet antigen 5, as well as one of the four yellow hornet antigen 5-specific antibodies, shown identical binding to the two hornet antigen 5s but negligible binding to wasp or yellowjacket antigen 5s. The remaining three yellow hornet antigen 5-specific antibodies, as well as the two yellowjacket antigen 5-specific antibodies, showed only significant binding of their autologous antigen 5s. The two wasp antigen 5-specific antibodies show very weak binding of the three homologous antigen 5s in addition to the expected binding of wasp antigen 5.

The degree of cross reactivity of vespid antigen 5s detected with monoclonal antibodies is much less than that detected with polyclonal antibodies. Apparently the majority of monoclonal antibodies which we had tested are specific for the unique epitopes of vespid antigen 5s, while the polyclonal antibodies are not. The findings with the monoclonal antibodies also provide evidence that the cross reactivity detected with polyclonal antibodies in Fig. 6 is not due to the possible cross-contamination of vespid antigen 5 samples, as the same samples were used for both assays with mono- or polyclonal antibodies.

Binding of white-faced hornet antigen 5-specific mouse antibodies to modified antigen 5s. Antibodies from two separate groups of mice immunized with antigen 5, in the presence of alum or complete Freund's adjuvant (groups A and B respectively),

were tested by enzyme immunoassay on solid-phase native or modified antigen. The modified antigen 5s include its reduced and carboxymethylated derivative and its CNBr cleavage fragments. The results in Fig. 6(A) and 6(B) were obtained with antibodies which were collected at week 13, late in the course of immunization, from groups A and B mice respectively. The antibodies which were obtained at week 7 early in the course of immunization from group A mice showed no detectable binding of the modified antigens (results not shown). The late antibodies from mice (group B) which were immunized with antigen 5 and complete Freund's adjuvant showed greater binding of the modified antigens than those (group A) immunized with antigen 5 and alum. For example the titers for one-third maximal binding of reduced and carboxymethylated antigen 5 are 6.5×10^1 and 3.5×10^1 respectively for the late antibodies of groups A and B at week 13, although their titers for the native antigen 5 are about the same, 2×10^5 .

The late antibodies from group B mice were purified by affinity chromatography on an immunosorbent containing native antigen 5. The purified antibodies showed binding activities for the native and the modified antigens which are identical to those of the unfractionated antibodies shown in Fig. 6(B). This finding suggests that the binding of late antibodies to modified antigens is not due to the presence of new populations of antibodies specific for the modified antigen, and that it is probably due to the increased affinity of the late antibodies for native or modified antigens. The progressive increase of antibody affinity during immune response is well known (Eisen, 1986).

The late antibodies from groups A and B mice showed nearly identical titers for native antigen 5 but this does not necessarily indicate that these antibodies had the same affinity. It is known that antibodies of high and low affinities can give identical binding curves on enzyme immunoassay because of the local high concn of solid-phase antigen (Berzofsky and Berkower, 1984; Steward and Lew, 1985).

DISCUSSION

The partial amino-terminal and carboxyl-terminal sequence data of vespid antigen 5s and their similar CNBr cleavage patterns provide chemical evidence on the structural homology of these proteins. There is a high degree of homology for the amino-terminal amino acid residues of these proteins, as 11–15 residues of their 20 residues compared are identical (Fig. 1).

The immunological data on the cross reactivity of vespid antigen 5s and the chemical data of vespid antigen 5s complement each other and suggest vespid antigen 5s to have homologous structures. Amino acid substitutions in homologous proteins generally occur on the surface of the molecule where the

antigenic sites are located (Benjamin *et al.*, 1984). Depending on the contribution of the particular amino acid residues to the antigenic sites, one may observe minor to major changes of the affinity of the homologous protein for the specific antibodies (Hannum and Margoliash, 1985). Complete structural data of vespid antigen 5s, when available, will be useful to analyze the amino acid residues which together form the antigenic sites of antigen 5.

Mouse antibodies specific for white-face hornet antigen 5 showed reduced binding of CNBr-cleaved antigen 5, or of reduced and carboxymethylated antigen 5, relative to that of native antigen 5. These results suggest that the two modified antigen 5s can retain or assume part of the conformation of the native molecule. This is of particular interest for the fully reduced and carboxymethylated derivative.

Vespid antigen 5s and scorpion neurotoxins are two groups of proteins of different sizes, containing about 190 and 70 amino acid residues respectively. Yet partial homology of these two groups of proteins is observed by comparing the amino-terminal 20 residues of yellowjacket antigen 5 with those residues of bark, European, and Mexican scorpion neurotoxins which are located at 9 or 19 residues away from the amino-terminal end; 7–8 residues of the 20-residue sequences compared, are identical (Fig. 2). Comparing the amino-terminal 20-residue sequences of yellowjacket antigen 5 and Brazilian scorpion neurotoxin, only three cysteinyl residues are identical (Fig. 2).

Many insect-allergic people have been treated with up to 100 µg amounts of vespid venoms without any ill effect (Lichtenstein *et al.*, 1979; Reisman, 1985). There is one isolated report that a patient developed transient but progressive bradycardia during immunotherapy with white-face and yellow hornet venoms (Lyanga *et al.*, 1982). We have immunized many mice i.p. with vespid antigen 5 (5–10 µg per mouse in the presence of adjuvant) with no ill effects but others have reported vespid venoms to be lethal in mice when given i.p. in larger amounts (about 300 µg per mouse) (Schmidt *et al.*, 1980).

A venom neurotoxin had been purified from an Asian hornet (*Vespa mandarina*). Its mol. size, charge, and amino acid composition are very similar to those of vespid antigen 5s, and it is reported to be a toxin specific for crustacean neuromuscular junctions (Abe *et al.*, 1982). Therefore the possibility of vespid antigen 5s being a neurotoxin of unknown specificity remains to be studied.

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