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Sensitivity of immunoassays for detecting cross-reactivity of homologous venom proteins of yellow jackets

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The venoms of yellow jackets of Vespula flavopilosa, V. pennsylvanica, V. squamosa, and V. vulgaris have similar proteins. Their major components comprise antigen 5, hyaluronidase, and phospholipase A₁. The homologous venom proteins share very similar biochemical properties. With the exception of antigen 5 and phospholipase of V. squamosa, they also have very similar antigenic properties. The venom proteins of V. squamosa and V. vulgaris were equally effective in inducing secondary antibody responses in mice that were primed with V. squamosa proteins. No cross-reaction of V. squamosa and V. vulgaris venom proteins was detected on immunodiffusion with specific mouse antisera. A very weak cross-reaction was detected by inhibition of ELISA, and a moderate cross-reaction was detected by direct ELISA. The varying cross-reactivity is a consequence of the different sensitivities of the assays. The sensitivity of the direct ELISA is apparently caused by a greater enhancement of the binding of low-affinity antibodies for the solid-phase antigen than that of high-affinity antibodies. Most untreated yellow jacket-sensitive patients tested had about tenfold higher levels of IgG specific for V. vulgaris proteins than those specific for V. squamosa proteins. This pattern of antibody specificity can be accounted for on the basis of cross-reactivity of these yellow jackets as suggested by the results with the mouse system. (J ALLERGY CLIN IMMUNOL 1987;79:113-20.)

For the diagnosis and treatment of insect allergy to hornets, yellow jackets, and wasps, it is useful to know the extent of immunologic cross-reactivity of their venom proteins. These insect venoms have similar protein compositions and are known to be allergens in man. Three of the venom proteins are hyaluronidase, phospholipase A₁, and antigen 5 of as yet unknown biochemical function. The homologous

proteins of these insects are found to have varying extents of cross-reactivity.¹⁻⁴

I will describe in this article the findings on the cross-reactivity of venom proteins of four species of yellow jackets, *Vespula flavopilosa*, *V. pennsylvanica*, *V. squamosa*, and *V. vulgaris*. They differ widely in their geographic distribution in North America.⁵ Other investigators have reported conflicting results on the cross-reactivity of these species of yellow jackets. One group reported that the venom of *V. squamosa* is distinct from the venoms of other species of yellow jackets when the venom is tested by immunodiffusion with rabbit antisera specific for *V. maculifrons* venom,⁶ but another group reported that the venom proteins of *V. squamosa* are antigenically re-

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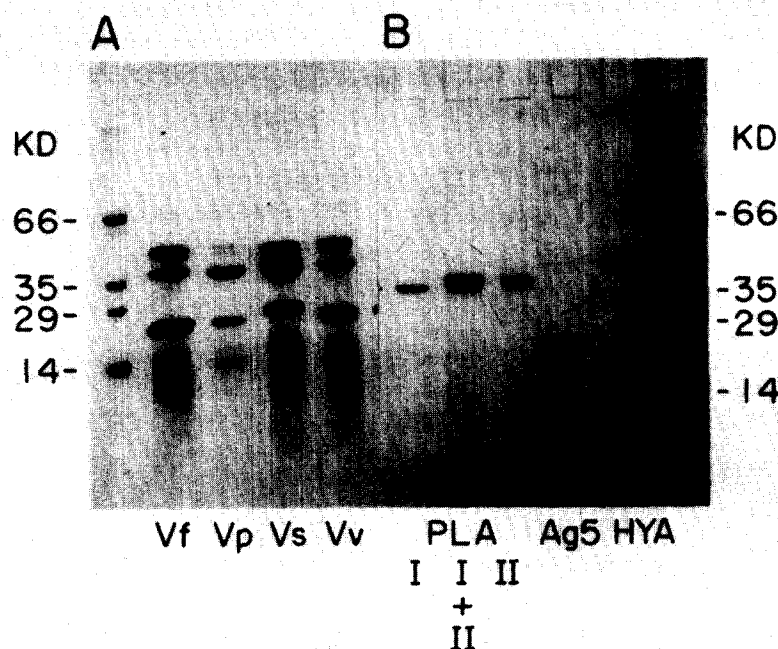


FIG. 1. Sodium dodecyl sulfate gel electrophoresis of yellow jacket venom proteins. **A**, A mixture of standard proteins. Venom sac extracts (left to right) of *V. flavopilosa*, *V. pennsylvanica*, *V. squamosa*, and *V. vulgaris*. About 24 μ g of each venom sac extract was used with the exception of *V. pennsylvanica*, of which 15 μ g was used. **B**, About 3 μ g each of purified venom proteins (left to right) of *V. squamosa* (from Fig. 2) in the order of phospholipase I, an admixture of phospholipase I plus II, phospholipase II, antigen 5, and hyaluronidase. All samples were reduced with mercaptoethanol before electrophoresis. The gel was stained with Coomassie blue R-250 dye.

lated to those of *V. maculifrons* when these venom proteins are tested by immunodiffusion with rabbit antisera specific for *V. squamosa* venom.⁷ Results obtained with sera from allergic humans by direct RAST or by RAST inhibition suggest that the venoms of *V. squamosa* and *V. pennsylvanica* can be distinguished from the venoms of other species of yellow jackets.⁶⁻⁸

We had previously demonstrated that the yellow jacket venom proteins of *V. germanica*, *V. maculifrons*, and *V. vulgaris* are immunochemically indistinguishable.² The present findings together with our earlier ones indicate that the venom proteins of most yellow jacket species are antigenically closely related, but two of the venom proteins of *V. squamosa* can be distinguished antigenically from the homologous proteins of other species of yellow jackets. The findings also indicate that direct binding test of antibodies on solid-phase antigens is more sensitive than immunodiffusion or inhibition tests in studying antigenic cross-reactivity.

MATERIAL AND METHODS

Venom sac extracts of yellow jackets of the species *V. flavopilosa*, *V. pennsylvanica*, *V. squamosa*, and *V. vul-*

garis were products of Vespa Laboratories, Spring Mills, Pa. The lot numbers of extracts used were 03038118 YJ, 03038117 YJ, 12228207 YJ, and 01038301 YJ, respectively. The procedures used for the isolation and characterization of venom proteins are cited in our previous articles.^{2,4} Affinity chromatography of phospholipase was carried out on an adsorbent containing its substrate analog that is 1-(11-carboxyundecyl)-2-hexadecyl-rac-glycero-3-phosphorylcholine.⁹ The weights of purified proteins were determined by amino acid analysis, and the weights of venom sac extracts were supplied by the manufacturer.

Mouse antibodies specific for the purified venom proteins were obtained from hyperimmunized BALB/c mice as follows: Groups of four mice were administered five biweekly intraperitoneal injections of venom proteins (2 μ g per mouse). A mixture of protein and alum was used for the first two injections, and a 9:1 mixture of complete Freund's adjuvant and protein solution¹⁰ was used for subsequent injections. Ascites were collected after week 10, but only in small amounts. At week 20 the mice were again each immunized with a mixture of protein and alum, and three days later each mouse was injected with 0.5×10^6 sp 2/0 cells.¹¹ About 50 ml of ascites were collected from each group during week 24 to 26.

Sera from untreated yellow jacket-sensitive patients in the Baltimore area were obtained from Dr. Anne Kagey-Sobotka of The Johns Hopkins University. The procedures

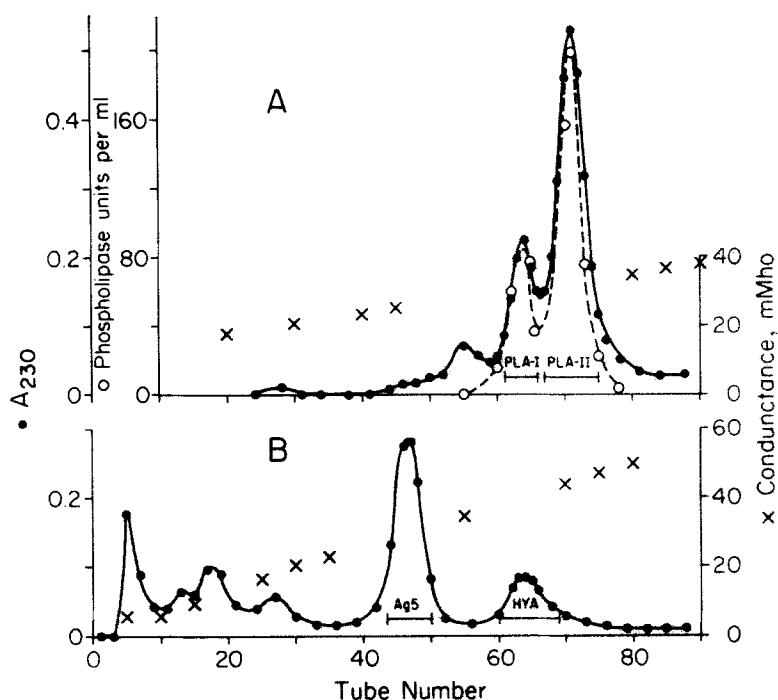


FIG. 2. Purification of *V. squamosa* venom proteins. **A**, Separation of the phospholipase-enriched venom fraction on a 14 by 0.9 cm column of SE-53. The column was eluted at 60 ml/hr with a linear NaCl gradient from 0.1 to 0.4 mol/L in 500 ml of 0.05 mol/L ammonium acetate buffer (pH 4.75), and tubes of 4 ml volume were collected. **B**, Separation of the phospholipase-depleted venom fraction on a 6 by 0.9 cm column of heparin-Sepharose. The column was eluted at 60 ml/hr with a linear NaCl gradient from 0 to 1 mol/L in 300 ml of 0.05 mol/L ammonium acetate buffer (pH 4.75), and tubes of 2 ml volume were collected. These two chromatograms were obtained with 91 mg of venom sac extract.

used for immunodiffusion, ELISA^{3,12} and ELISA inhibition by a competing antigen were described previously.⁴ The specificity of immunoassay procedures and reagents was established with unrelated antigens and antibodies.

Results

The venom sac extracts of *V. flavopilosa*, *V. pennsylvanica*, *V. squamosa*, and *V. vulgaris* have very similar protein compositions, as indicated by their sodium dodecyl sulfate gel electrophoretic patterns (Fig. 1, A). The three major proteins of *V. vulgaris* venom, with molecular weights of about 23, 35, and 47 kilodaltons, were previously identified to be antigen 5, phospholipase A₁, and hyaluronidase, respectively.² The protein bands of *V. pennsylvanica* are less intense than those of other yellow jackets because the amount of *V. pennsylvanica* venom applied to the gel is lower than those of other venoms. These four yellow jacket species were found to have nearly identical levels of hyaluronidase and phospholipase activities in their venom sac extracts.

The three major proteins of *V. squamosa* venom were isolated as follows: Phospholipase was isolated from venom by affinity chromatography on an adsor-

bent containing its substrate analog followed by ion exchange chromatography on SE-53 cellulose. Two isoelectric forms of phospholipase, I and II, with identical specific activities were obtained (Fig. 2, A). The two purified phospholipases differed in their gel electrophoretic mobilities (Fig. 1, B) just as they did in the venom mixture (Fig. 1, A). Antigen 5 and hyaluronidase were isolated from the phospholipase-depleted venom by chromatography on heparin-Sepharose (Fig. 2, B), and they were each rechromatographed on Sephadex G-100 to remove trace contaminants. The purified antigen 5 gave a single band on gel electrophoresis, but the hyaluronidase gave a doublet just as the venom did (Fig. 1, B).

The yields and biochemical properties of the purified proteins from *V. squamosa* are presented in Table I together with those of the proteins from *V. vulgaris*. Similar findings were obtained for these two species of yellow jackets. The only notable difference is the presence of two isoelectric forms of phospholipase in *V. squamosa* venom, whereas there is only one form in *V. vulgaris*. The amino acid compositions of antigen 5 from *V. squamosa* and *V. vulgaris* were found to be similar as was also the case

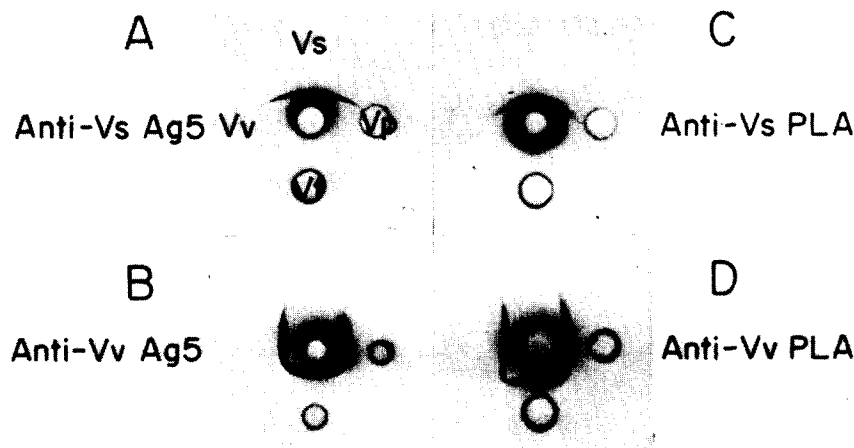


FIG. 3. Immunodiffusion of venom proteins of different yellow jackets. **A-B,** The center wells were filled with mouse antisera specific for antigen 5s of *V. squamosa* and *V. vulgaris*, respectively. The four peripheral wells, anticlockwise starting from the top, were filled with *V. squamosa* antigen 5 (0.15 mg/ml), *V. vulgaris* antigen 5 (0.13 mg/ml), and *V. flavopilosa* and *V. pennsylvanica* venoms (5 mg/ml), respectively. **C-D,** The arrangements are the same as in **A** and **B** except that phospholipase-specific sera and purified phospholipases (0.12 to 0.25 mg/ml) were used. Two 10 μ l additions of antigen and antisera solutions were made at 0 and 16 hours, and the gels were stained after 40 hours.

TABLE I. Yields and properties of venom proteins of *V. squamosa* and *V. vulgaris*

	Yield per 100 mg venom sac extract	A_{280} for c = 1 mg/ml	Specific activity (unit/milligram)
Antigen 5			
<i>V. squamosa</i>	0.8	2.0	na
<i>V. vulgaris</i>	0.7	1.9	na
Hyaluronidase			
<i>V. squamosa</i>	0.3	nd	6000
<i>V. vulgaris</i>	0.2	1.9	5900
Phospholipase			
<i>V. squamosa</i>	2.3	0.97	2100
<i>V. vulgaris</i>	2.0	0.89	1200

A_{280} = absorbance at 280 nm; na = not applicable; nd = not determined.

The yield of antigen 5 from *V. vulgaris* was about one half of that reported.^{2,4} The specific activity of hyaluronidase and phospholipase was also about half of that reported previously. The yield of phospholipase for *V. squamosa* is the combined amount of isoelectric forms I and II that were obtained in a ratio of about 1 to 3.

for their homologous phospholipases (results not presented).

Antisera specific for antigen 5s and phospholipases of *V. squamosa* and *V. vulgaris* were obtained from ascitic fluids of hyperimmunized mice. The specificity of each of the antisera was ascertained by immunodiffusion with venoms or purified venom proteins other than those used as immunogens (results not presented). On immunodiffusion experiments demonstrated that each antiserum formed a strong precipitin line only with its immunogen, as demonstrated in Fig. 3. There is one exception in that *V. squamosa* antigen 5 formed a fuzzy line with *V. vulgaris*-specific

antiserum. The venoms of *V. flavopilosa* and *V. pennsylvanica* each formed a precipitin line with antiserum specific for *V. vulgaris* antigen 5 or phospholipase but not with antisera specific for *V. squamosa* proteins.

Mouse antiserum specific for *V. vulgaris* hyaluronidase was also prepared. When that serum sample was used for immunodiffusion with the purified hyaluronidases of *V. squamosa* and *V. vulgaris*, and with the venoms of *V. flavopilosa* and *V. pennsylvanica*, a single precipitin line was formed in each case, and complete identity of the precipitin lines of these samples was observed. These results are not presented.

The immunodiffusion results in Fig. 3 illustrate that

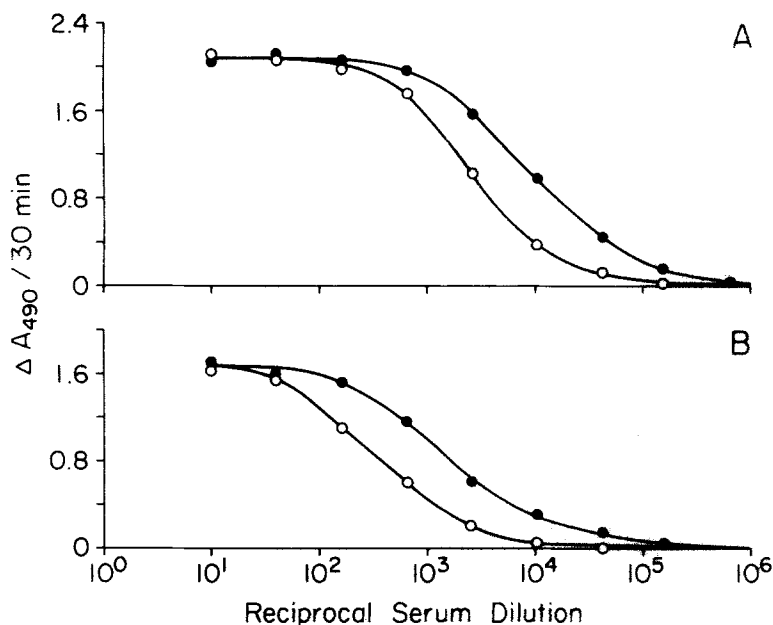


FIG. 4. Enzyme immunoassay of mouse antisera specific for *V. squamosa* proteins on solid-phase *V. squamosa* (filled circles) or *V. vulgaris* (open circles) proteins. **A**, Antisera specific for *V. squamosa* antigen 5 were assayed on solid-phase *V. squamosa* or *V. vulgaris* antigen 5. **B**, Antisera specific for *V. squamosa* phospholipase were assayed on solid-phase *V. squamosa* or *V. vulgaris* phospholipase.

the mouse antisera specific for *V. squamosa* antigen 5 or phospholipase did not bind the homologous proteins of *V. vulgaris*. However, when these same serum samples were tested by ELISA on solid-phase *V. vulgaris* proteins, binding was detected. The difference in concentrations of antisera required for half maximal binding in Fig. 4, A, suggest that the mouse antiserum specific for *V. squamosa* antigen 5 bound solid-phase *V. vulgaris* antigen 5 about five times less tightly than the mouse antiserum bound the solid-phase *V. squamosa* antigen 5. This is also the case for the mouse antiserum specific for *V. squamosa* phospholipase (Fig. 4, B). Mouse antisera specific for *V. vulgaris* antigen 5 or phospholipase were found to bind similarly solid-phase *V. squamosa* proteins; these results are not presented.

The mouse antiserum samples specific for *V. squamosa* antigen 5 or phospholipase were also tested for their binding to *V. squamosa* or *V. vulgaris* proteins by inhibition of enzyme immunoassay, that is, *V. squamosa* or *V. vulgaris* proteins were used to inhibit the binding of antibodies to solid-phase *V. squamosa* proteins. The difference in concentrations of *V. squamosa* and *V. vulgaris* proteins required for 50% inhibition in Fig. 5, A and B, suggest that the mouse antibodies specific for *V. squamosa* antigen 5 or phospholipase bound the *V. vulgaris* proteins about 10^4 fold less tightly than they bound the *V. squamosa* proteins.

Another test of the cross-reactivity of *V. squamosa* and *V. vulgaris* proteins was to compare their efficiencies to induce secondary antibody responses in mice that were primed with *V. squamosa* proteins. The results in Table II illustrate that *V. vulgaris* proteins were just as efficient as *V. squamosa* proteins were in stimulating secondary antibody responses specific for *V. squamosa* antigen 5 or phospholipase. The results in Table II also confirm the antigenic individuality of these two species of yellow jackets, since the mice that were boosted with *V. vulgaris* proteins evoked larger *V. vulgaris*-specific antibody responses than those mice that were boosted with *V. squamosa* proteins.

We also measured specific IgG levels in sera of nine untreated yellow jacket-sensitive patients by enzyme immunoassay on solid-phase antigen 5s and phospholipases from *V. squamosa* and *V. vulgaris*. The results from one representative patient are presented in Fig. 6, and those of all patients are summarized in Table III. Most patients tested had higher levels of IgG specific for *V. vulgaris* proteins than those specific for *V. squamosa*. The differences in IgG levels specific for these two yellow jackets were much less than tenfold with the single exception of patient 12. This pattern of antibody specificity can be accounted for on the basis of cross-reactivity of these two yellow jackets, as suggested by the results in the mouse system.

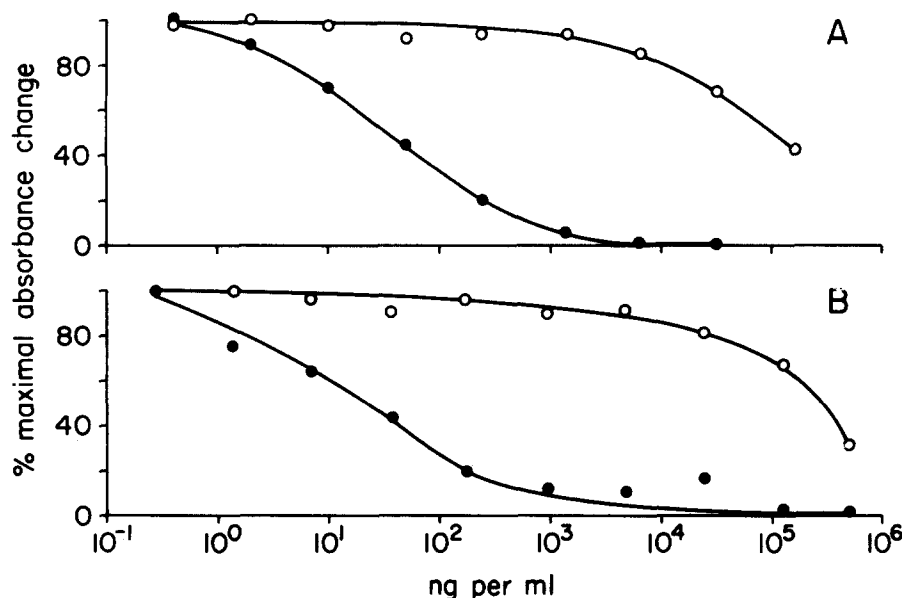


FIG. 5. Inhibition of enzyme immunoassay of mouse antisera specific for *V. squamosa* proteins on solid-phase *V. squamosa* proteins by *V. squamosa* (filled circles) or *V. vulgaris* (open circles) proteins in solution. A, Antigen 5 and antigen 5-specific serum samples were used. B, Phospholipase and phospholipase-specific serum samples were used. In both A and B, the serum samples were used at a dilution corresponding to one third maximal binding to solid-phase protein.

DISCUSSION

The above findings with mouse antisera demonstrate antigenic cross-reactivity of the venom proteins of the four species of yellow jackets studied in this article. They also demonstrate that antigen 5 and phospholipase of *V. squamosa* have distinctive antigenic features when these are compared to the homologous proteins of other yellow jackets. The antigenic relationship of these yellow jacket venoms may reflect their taxonomy because the yellow jackets in North America are classified by entomologists into separate species groups.⁵ *V. Squamosa* belongs to one species group, and all the others that we have studied belong to a separate group.

The cross-reactivity of *V. squamosa* and *V. vulgaris* proteins is also supported by the serologic findings of untreated yellow jacket-sensitive patients. These patients demonstrated similar levels of IgG specific for *V. squamosa* and *V. vulgaris* proteins, and it is unlikely that, of the nine patients tested in Table III, they all have been sensitized to both species of yellow jackets.

The cross-reactivity data on specific IgG of patients in Table III are applicable to their specific IgE as well, since we have demonstrated previously that vespid-allergic patients invariably had IgE and IgG of the same specificity.^{2,3} The present findings together with those of other studies^{2,6-8} strongly suggest that the venom proteins of eight common species of yellow

jackets, which have been studied thus far, are antigenically and allergenically the same or closely related.

Varying extents of antigenic cross-reactivity of *V. squamosa* and *V. vulgaris* antigen 5s, and of their phospholipases were observed by use of three different assay procedures. No cross-reactivity was observed on immunodiffusion, very poor cross-reactivity by inhibition of ELISA, and moderate cross-reactivity by direct ELISA. The varying results must reflect the sensitivities of the assays, since exactly the same reagents were used.

The binding of antigen and antibody occurs in different media with the three methods used for detection of cross-reactivity. For immunodiffusion the reaction occurs in gel. For inhibition of enzyme-linked immunoassay it occurs in solution. In enzyme-linked immunoassay, the antibodies in solution bind the solid-phase antigens. It is known that immunodiffusion is an insensitive method for detecting antibodies of low affinity in addition to the requirement of multisite reactions of antigens with bivalent antibodies in order that immune precipitate can form. This is in contrast to enzyme-linked immunoassay in which antibody-antigen binding by a single site is sufficient and in which there is an enhancement of the antibody affinity for antigens attached to solid phase, such as cell surface or walls of plastic microtiter plates.¹³

Two factors have been indicated for the enhance-

TABLE II. Antibody responses in mice primed with *V. squamosa* protein and then boosted with *V. squamosa* or *V. vulgaris* protein

Group	Immunogen used		Vs-specific Ab titer		Vv-specific Ab titer	
	Week 0	Week 3	Week 3	Week 6	Week 3	Week 6
A	Vs Ag5	Vs Ag5	420	4,500	24	290
B	Vs Ag5	Vv Ag5	480	20,000	24	5600
C	Vs PLA	Vs PLA	2400	12,000	310	1800
D	Vs PLA	Vv PLA	2600	9,100	390	5600

Vs = *V. squamosa*; Vv = *V. vulgaris*; Ab = antibody; Ag 5 = antigen 5; PLA = phospholipase.

Groups of four female BALB/c mice were immunized intraperitoneally with 0.2 ml of a mixture of the venom protein (2 µg) and alum (1 mg) in phosphate-buffered saline (pH 6.4) as indicated. The collected sera for each group were pooled, and the antibody titers were determined in duplicate by enzyme immunoassay on solid-phase antigen 5 or phospholipase as presented in Fig. 4. The titers presented correspond to the reciprocal serum dilution required for one third of maximal binding.

Group A mice had a lower antibody titer for *V. squamosa* antigen 5 at week 6 than group B mice had. This was unexpected because group A mice were boosted with *V. squamosa* antigen 5, whereas group B mice were boosted with *V. vulgaris* antigen 5.

TABLE III. Specific IgG in sera of nine untreated yellow jacket-sensitive patients

Patient No.	Antigen 5-specific IgG		Phospholipase-specific IgG	
	<i>V. vulgaris</i>	<i>V. squamosa</i>	<i>V. vulgaris</i>	<i>V. squamosa</i>
1	740	1100	2000	230
7	140	60	500	80
8	170	170	1100	1100
9	350	150	180	600
12	500	400	500	30
16	160	300	300	500
18	350	100	700	500
19	70	40	180	30
20	100	80	580	580

Specific IgG was measured by enzyme immunoassay on solid-phase antigen 5 or phospholipase as presented in Fig. 6, and the values represent the reciprocal serum dilutions required to elicit one third of maximal binding. Normal persons who were not allergic to vespids had negligible titers of vespid-specific IgG.^{2,3}

ment of antibody affinity in solid-phase immunosorbent assays.¹³ One factor is that the high effective local concentration of antigen on the solid phase suppresses the dissociation of antigen-antibody complexes. Another factor is that multisite interaction of antigen-antibody molecules can occur more effectively on the solid phase than it can in solution. Such multisite interaction will greatly enhance the apparent affinity of antibody for antigen. For example, the functional affinity of bivalent IgG for solid-phase multivalent hapten is about 10^3 -fold larger than its intrinsic affinity for monovalent hapten in solution.¹⁴ By contrast, the enhancement of functional affinity for multivalent antibody-antigen interaction in solution is $<10^2$ -fold larger than that of monovalent antigen-antibody interaction in solution.¹⁵

By inhibition of ELISA, the mouse antibodies specific for *V. squamosa* proteins bound the *V. vulgaris* proteins about 10^4 -fold less tightly than they bound

the *V. squamosa* proteins, but by direct ELISA, the difference is only about fivefold. These results suggest that there is a greater enhancement of the binding of low-affinity antibodies for solid-phase immunosorbent than for that of high-affinity antibodies. An explanation for this difference is still forthcoming, but it appears to be related to the epitope density of the immunosorbent. For example, low-affinity DNP-specific antibodies bound poorly to solid-phase bovine plasma albumin with low degree of DNP substitution while high-affinity antibodies bound well, and yet both high- and low-affinity antibodies bound equally well to solid-phase albumin heavily substituted with DNP groups.^{16,17}

The number of amino acid substitutions at individual antigenic sites that are required for the observed level of cross-reactivity of venom proteins of *V. squamosa* and *V. vulgaris* will only be known when structural knowledge of the antigenic sites of these proteins

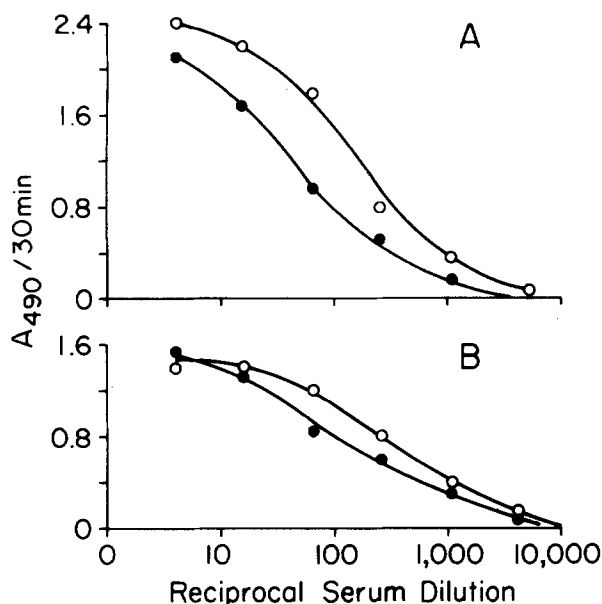


FIG. 6. Enzyme immunoassays for antigen 5 and phospholipase-specific IgG in serum of an untreated yellow jacket-sensitive patient. **A.** The serum sample was assayed on solid-phase *V. squamosa* (filled circles) and *V. vulgaris* (open circles) antigen 5s. **B.** The serum sample was assayed on solid-phase *V. squamosa* (filled circles) and *V. vulgaris* (open circles) phospholipases.

becomes available. The cross-immunization data in Table II suggest that the structural differences of the homologous proteins of *V. squamosa* and *V. vulgaris* are not extensive. If this were not so, the proteins of *V. vulgaris* would not have been as efficient as the homologous proteins of *V. squamosa* were to induce secondary antibody responses specific for *V. squamosa*.

We have previously observed that there is limited cross-reactivity of the antigen 5 and phospholipase proteins of hornets, yellow jackets, and wasps when these proteins are tested by inhibition assay.⁴ When these homologous proteins were retested by direct enzyme immunoassay with the same mouse antisera used previously, higher degrees of cross-reactivity were observed (unpublished results). Hirayama et al.¹⁸ found that one of their antilysozyme peptide monoclonal IgM antibodies reacted about equally well with lysozyme or its peptide by direct enzyme immunoassay. When cross-reactivity was tested by inhibition assay, antibody binding of peptide with an affinity constant of $5 \times 10^4 \text{ mol/L}^{-1}$ was observed, but antibody binding of lysozyme was not detectable. Therefore, the greater sensitivity of direct enzyme immunoassay to detect cross-reactivity of related proteins and peptides is of general applicability.

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