

# Allergens in Hymenoptera venom

## XVIII. Immunoblotting studies of venom allergens

Donald R. Hoffman, Ph.D. Greenville, N. C.

*Pure venoms from yellow jackets and bees were used to evaluate denaturing and nondenaturing immunoblot techniques and to compare results of IgE antibody-stained immunoblots to RAST with highly purified allergens. Significant differences in IgE antibody binding were observed among all three techniques, suggesting that tertiary conformation is a major factor in the allergenic determinants of venom allergens. IgG antibodies from hyperimmunized animals and from specimens obtained from allergic patients before and after venom immunotherapy appeared to exhibit less conformational dependence for binding. The conformational dependence of IgE antibody binding to bee and yellow jacket phospholipases was confirmed by direct and inhibition RAST studies. The allergens studied were all single polypeptide chains. (J ALLERGY CLIN IMMUNOL 1987;80:307-13.)*

In our investigations of the allergens in *Solenopsis invicta* (imported fire ant) venom,<sup>1</sup> we observed that IgE binding was greatly reduced in nitrocellulose blots prepared from SDS-PAGE gels compared to blots prepared from either nonequilibrium pH gradient electrophoresis gels or nondenaturing acidic gels. Insect-venom allergens are generally very basic proteins, and they can not be separated or studied by many of the methods commonly used for acidic and neutral proteins.<sup>2</sup> For these reasons we were suspicious about the reliability of immunoblot studies with insect-venom allergens. Two relatively well-characterized venoms were used to study immunoblot techniques and to compare them with other methods for measuring IgE binding. We report in this article on studies with venoms from *Vespula maculifrons*, the Eastern yellow jacket, and *Apis mellifera*, the honeybee. The allergens of both these venoms are well studied, and the major allergens have been isolated and characterized.<sup>3-10</sup>

### MATERIAL AND METHODS

#### Venom and venom components

Honeybee venom was purchased from Champlain Valley Apiaries, Middlebury, Vt. Honeybee proteins were isolated

#### Abbreviations used

PAGE: Polyacrylamide gel electrophoresis  
SDS: Sodium dodecyl sulfate  
Tris: Tris(hydroxymethyl)aminomethane  
IEF: Isoelectric focusing

as previously described.<sup>7-9</sup> Venom from *V. maculifrons* workers was obtained by carefully squeezing sacs dissected from frozen insects, as has been described for other species.<sup>6</sup> The venom proteins were separated and purified by the methods we have described for *V. squamosa* venoms. The venom proteins used in this study were of higher purity than proteins isolated from venom sac extract,<sup>5</sup> and when the proteins were analyzed by SDS-PAGE, enzyme activities and immunochemical techniques were comparable to those reported for other vespid venoms.<sup>6</sup> The *V. maculifrons* hyaluronidase contained about 0.8% phospholipase, the phospholipase less than 0.1% hyaluronidase, and the antigen 5 less than 0.1% phospholipase and hyaluronidase by enzyme activity measurements. Hyperimmune antisera were raised by the use of Freund's complete adjuvant in rabbits<sup>5</sup> or guinea pigs.<sup>2</sup>

#### Human sera

Sera from patients with recent histories of systemic reactions to the appropriate insect were used as sources of IgE antibodies. Except where it is specified, all sera were obtained before any venom immunotherapy. Postimmunotherapy sera were collected when the patient reached the top dose (usually 100 µg for single species or 300 µg for mixed vespid venom-protein extract) and full interval. All sera were RAST positive to the appropriate venom, and all patients were also skin test positive to the same venom.

From the Department of Pathology, East Carolina University School of Medicine, Greenville, N. C.

Received for publication March 10, 1986.

Accepted for publication March 7, 1987.

Reprint requests: Donald R. Hoffman, Ph.D., Department of Pathology, East Carolina University School of Medicine, Greenville, NC 27858.

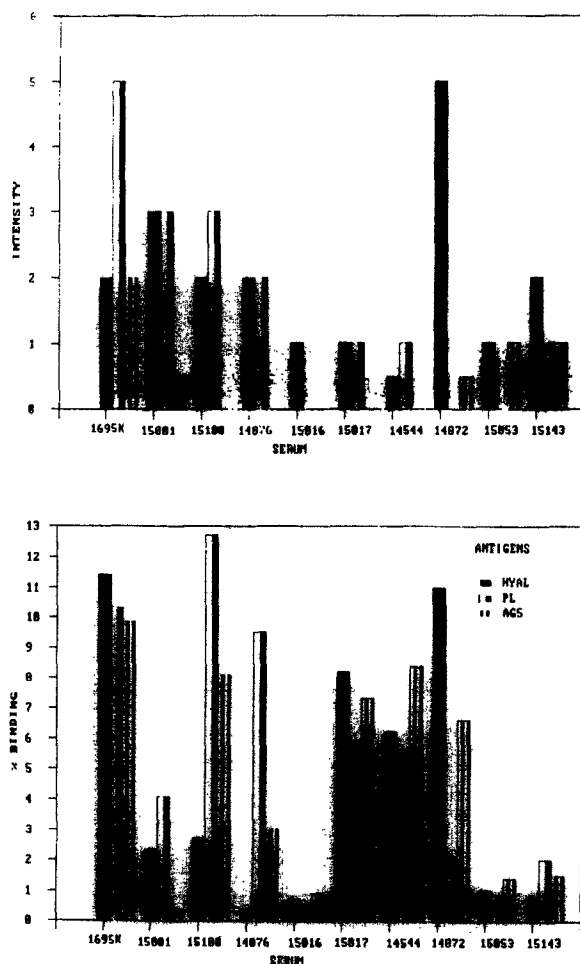


**FIG. 1.** Comparison of IgE antibody binding to *V. maculifrons* yellow jacket-venom allergens in an immunoblot prepared from a nondenaturing acid PAGE gel (top) to IgE antibody binding to purified allergens measured by RAST (bottom). The immunoblot was stained with a biotin-streptavidin amplified horse radish peroxidase method. Relative intensities of stain were measured by reflectance densitometry. RAST was performed with disks coupled at limiting dilution and is expressed as percent of total activity added bound to the disk minus nonspecific binding for that allergen disk.

Sera were chosen for this study to represent a variety of patterns of cross-reactivity and were not randomly chosen or representative of a random population.

### Electrophoresis

The methods used for SDS-PAGE, nondenaturing acidic gels, and nonequilibrium pH gradient electrophoresis in minigels have been previously described in detail.<sup>1</sup> Venoms were applied to gels at 4  $\mu$ g of protein per centimeter of gel width.

### Transfer blots

All transfers were made by transverse electrophoresis onto Whatman WCN nitrocellulose membrane (Curtin

Matheson Scientific Inc., Marietta, Ga.). Use of the standard Tris-glycine buffer with 20% methanol<sup>11</sup> did not provide good transfers from SDS-PAGE with venom allergens, but use of 0.025 mol/L of ethanolamine/glycine with 20% methanol elicited much better transfer.<sup>12</sup> Blots from nondenaturing gels were transferred in 0.7% acetic acid. Nitrocellulose membranes were blocked with 5% bovine serum albumin in Tris-buffered saline, pH 7.4, before immunodetection.

### Immunodetection on nitrocellulose blots

The immunodetection protocols used were as previously described by our laboratory.<sup>1</sup> Human IgG antibodies were detected with biotinylated antihuman IgG and streptavidin-horse radish peroxidase (Bethesda Research Laboratories, Gaithersburg, Md.). Rabbit IgG antibodies were detected with antirabbit IgG-horse radish peroxidase and protein by biotinylation and staining with avidin-horse radish peroxidase (Bio-Rad Laboratories, Richmond, Calif.). Peroxidase-stained strips were scanned on a computerized densitometer (Hoefer Scientific Instruments, San Francisco, Calif.). Sera from nonallergic individuals exhibited no IgE binding to blots prepared from bee and yellow jacket venoms.

### RAST

RAST was performed as previously described<sup>13</sup> with purified allergens by coupling the cyanogen bromide-activated paper disks with limiting quantities of antigen, 0.2  $\mu$ g of protein per disk, to maximize specificity, as has been described.<sup>14, 15</sup>

### RESULTS

Pure *V. maculifrons* venom was separated by SDS-PAGE after reduction by dithiothreitol and heating at 100° C for 5 minutes and then electrophoretically transferred to nitrocellulose membranes in ethanolamine/glycine buffer containing 20% methanol. Native preparations of *V. maculifrons* venom were separated by acidic gel PAGE and nonequilibrium IEF. Good separations were obtained by both nondenaturing systems, but the resolution was higher in the acidic buffer gels than in nonequilibrium IEF. Antigens were transferred to nitrocellulose electrophoretically in 0.7% acetic acid from nondenaturing gels. The positions of the component allergens of the venom were determined by electrophoresis of purified components and also by immunostaining with hyperimmune rabbit antisera raised against the purified components. The presence of each allergen on the transfer blots was demonstrated by both immunostaining with rabbit antibody and by protein detection on the blots with biotinylation followed by detection with avidin-peroxidase.

Strips cut from a transfer blot prepared from a nondenaturing acidic PAGE gel were incubated with 1:4 dilutions of individual pretreatment sera from patients

allergic to yellow jacket venom. The specificity patterns of the sera were chosen to represent patterns commonly encountered. Four patients reacted in RAST with only yellow jacket, three other patients reacted with all the vespids, and three additional patients reacted with honeybee and vespids. IgE against *V. maculifrons* allergens was detectable in all the sera by both immunoblot and RAST. The results with these two techniques are compared in Fig. 1. The blots were developed with a biotin-streptavidin—amplified system, and detection was by peroxidase. Reactivities were quantitated from a densitometric scan. As can be observed in Fig. 1, most of the sera contained IgE antibodies reacting with hyaluronidase and phospholipase, whereas only five sera contained IgE antibodies reacting with antigen 5. All 10 sera contained IgE antibodies reacting with antigen 5 by RAST. Quantitatively, there was little correlation between the allergen reactivity pattern observed in the nondenaturing immunoblot and that observed in RAST. Linear correlation coefficients for the RAST with purified allergens were 0.21 for hyaluronidase and phospholipase, 0.78 for hyaluronidase and antigen 5, and 0.64 for phospholipase and antigen 5, suggesting that the reactions are independent. Therefore, the difference in results can not be attributed to cross-contamination of the antigens used for RAST. Several additional sera, which were primarily reactive with antigen 5 by RAST, were tested in immunoblot analysis and elicited only weak binding to antigen 5.

In further experiments, it was found that IgE binding to antigen 5 could be increased by use of less dilute sera. In one experiment, there was no detectable binding at 1:10, a trace of binding at 1:5, and strong binding at 1:2. In the same experiment, binding to phospholipase was strong at all three concentrations. Antigen 5 was readily detectable on all blots by both immunostaining with hyperimmune rabbit IgG and protein detection by biotinylation.

The binding of human IgG antibodies to *V. maculifrons* antigens separated by SDS-PAGE is illustrated in Fig. 2. Densitometer scans from two sets of paired sera before and after venom immunotherapy are presented. The patterns illustrated are representative of patterns obtained from the 17 pairs of sera tested. IgG antibodies were detected that bound to all the five known allergens of *V. maculifrons*.<sup>5</sup> Significant increases in IgG antibody levels occurred as a result of immunotherapy. Interestingly, antibody of new specificities rarely developed as a result of therapy. Almost all the changes observed in both IgE and IgG were increases of the specificities already present. There was no difficulty in detecting IgG antibody binding to antigen 5 on immunoblots, as was observed with IgE binding.

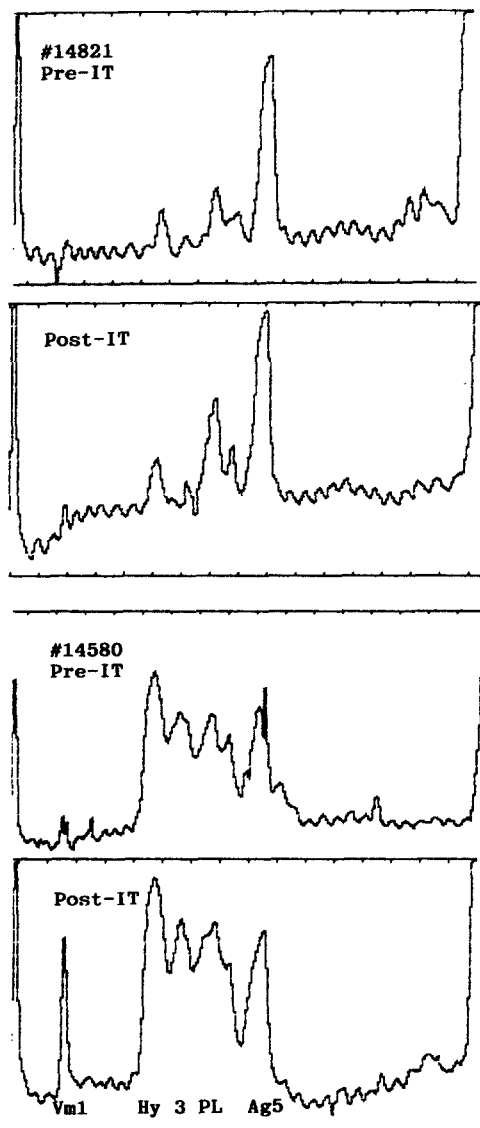


**FIG. 2.** Densitometric scan of immunoblots prepared from SDS-PAGE gels of *V. maculifrons* venom. Strips were incubated with human serum obtained before immunotherapy and at maintenance and were stained for human IgG by an immunoperoxidase method. The scans of two sets of paired sera are presented. The locations of the five known venom allergens are indicated on the *bottom* scan. The ordinate of the scans is dye intensity and the abscissa is distance, with the sample applied at the *left*. A significant increase in IgG antibodies can be observed as a result of therapy.

To investigate further the possible role of conformational effects on IgE antibody binding, matched gels were run in SDS denaturing and acidic gel non-denaturing systems, and strips from each were incubated with a panel of 14 sera from yellow jacket venom-allergic patients. The results are illustrated in Fig. 3. In order to obtain maximum sensitivity and resolution, the strips were incubated in <sup>125</sup>I-labeled

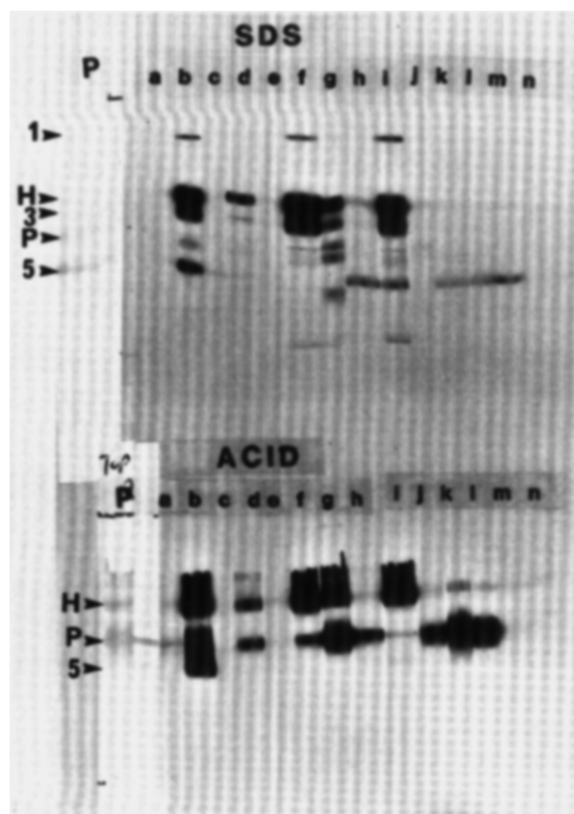


**FIG. 3.** Autoradiographs of SDS-PAGE (*top*) and non-reducing acid PAGE gel (*bottom*) immunoblots developed with  $^{125}\text{I}$ -labeled anti-IgE. The same 14 sera from allergic patients were used for both experiments. The strips on the *left* are cut from the immunoblots and stained for protein by biotinylation and avidin-peroxidase. The positions of the venom proteins are indicated: H, hyaluronidase; P, phospholipase; 1, Vmac1; 3, Vmac3; 5, antigen 5. Both experiments were performed under the same conditions, and the exposures illustrated were made on the same piece of film.

anti-IgE and developed by autoradiography. Binding was generally lower to the blots prepared from the SDS-PAGE gel, although more components were resolved by this gel. Binding was observed for all sera in the acid gel blots and for 13 of 14 sera in the SDS-PAGE blots. Binding to antigen 5 was only visible for five sera on the acid blots but was observed in eight sera on the SDS-blots. However, binding to phospholipase was much more prevalent and of greater intensity on the acid gel blots than on the SDS-PAGE blots. Binding to hyaluronidase was strong on both types of blot. Binding to Vmac1 could only be observed on SDS-PAGE blots, probably because it does not migrate into the acid gel. Vmac3 did not fully separate from hyaluronidase in the acid gel.

Studies were also carried out with honeybee venom

with the acidic gel system for separation. It was possible to find all five known honeybee allergens, including the two that do not have a basic charge on nitrocellulose blots, with hyperimmune rabbit and guinea pig antisera. Blots prepared from honeybee venom were tested with sera from allergic individuals known to have a variety of allergen reaction patterns, including several patterns that appeared to be mono-specific for single allergens by RAST,<sup>7-9</sup> as are illustrated in Fig. 4. Most sera known to be reactive with acid phosphatase, hyaluronidase, or phospholipase demonstrated IgE binding to these bands. Only about half of the sera that reacted to allergen C by RAST reacted by immunoblot. Very little reactivity was observed with melittin, even with sera that reacted strongly by RAST. In addition, binding to melittin by human IgG antibodies was also very weak. IgG antibodies were readily detectable in sera both before and after venom immunotherapy against the other four allergen bands.

The conformational dependence of IgE binding to bee and vespid phospholipases was directly tested by RAST and RAST inhibition with pools of sera from allergic patients. The RAST-inhibition results for reduced and alkylated phospholipases are illustrated in Fig. 5. The relative inhibition constant for *V. maculifrons* phospholipase decreased 300-fold after treatment with 0.1% SDS and dithiothreitol, and by 700-fold when this treatment was followed by immersion for 5 minutes in boiling water, as in the sample manipulation for SDS-PAGE. The cyanogen bromide digest of *V. maculifrons* phospholipase had no measurable direct binding of IgE antibodies from sera of allergic individuals. Reduction and alkylation of this phospholipase without denaturant caused a 430-fold decrease in inhibitory capacity, and there was no measurable activity if the phospholipase was reduced and alkylated in the presence of 8 mol/L of guanidine HCl. Honeybee phospholipase demonstrated a 75-fold reduction in inhibitory capacity after mild reduction and alkylation.

## DISCUSSION

Immunoreactive nitrocellulose blots were prepared from reduced and boiled SDS-PAGE, nonequilibrium IEF, and nondenaturing acidic PAGE gels with venoms from *V. maculifrons* and *A. mellifera*. Significant differences were observed in the immunoreactivity of the individual venom allergens with human IgE antibodies in sera from allergic patients, especially for antigen 5 and phospholipase from *V. maculifrons* and allergen C from *A. mellifera*. A panel of 10 allergic sera was tested both by RAST with highly purified

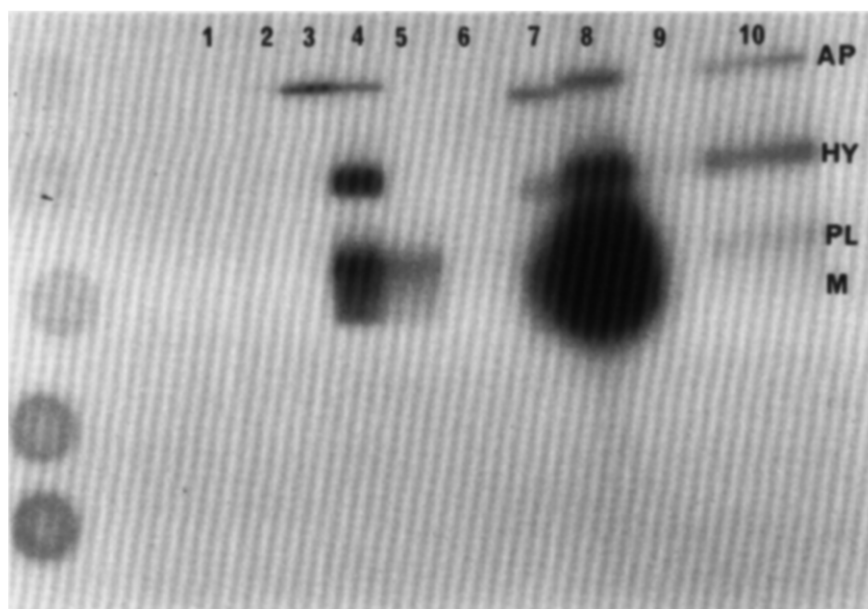


**FIG. 4.** IgE immunoblot prepared from nondenaturing acidic gel electrophoresis of honeybee venom. The positions of the bee-venom proteins as determined by electrophoresis of purified proteins and immunostaining with monospecific antisera are illustrated on the *right*. AP, acid phosphatase; HY, hyaluronidase; PL, phospholipase A; M, melittin. The strips were each incubated with serum from a single patient. Serum 1 was a negative control, sera 2 and 3 reacted by RAST with acid phosphatase and allergen C, sera 4 and 10 reacted primarily to hyaluronidase by RAST, sera 5 and 8 were primarily phospholipase reactive by RAST, serum 7 reacted with all the bee-venom allergens by RAST, and sera 6 and 9 were only weakly reactive by RAST. The *five circles on the left* are radioactive exposure standards.

allergens coupled at limiting dilution and by nondenaturing acid gel PAGE, and a low correlation between quantitative allergen reactivity patterns was observed (Fig. 1). The correlation between RAST and denaturing SDS-PAGE was even less. By contrast, hyperimmune rabbit IgG antibodies and, to some degree, human IgG antibodies (Fig. 2) bound strongly to immunoblots prepared from both denaturing and nondenaturing gels. IgE binding to antigen 5 demonstrated dependence on serum concentration, with binding observed only at high concentrations of serum. This suggests that the low reactivity of IgE antibodies with antigen 5 is a result of low affinity and not of blocking by IgG antibodies. In RAST assays, IgG blocking effects are usually greater at higher serum concentrations.

The most probable explanation for the lack of correlation between RAST, nondenaturing blots, and denatured blots is the dependence of IgE antibody binding on antigen conformation. Benjamin et al.<sup>16</sup> have proposed that most antigenic determinants are topographic in nature rather than a linear sequence of amino acids. These topographic determinants would be minimally preserved in blots prepared from

SDS-PAGE in which the proteins have been reduced and the tertiary structure destroyed by SDS binding. Some of these topographic determinants could be changed in the interactions necessary for the strong binding of proteins to nitrocellulose, probably by hydrophobic binding. The allergen in the RAST assay is probably close to native conformation, since it is just bound at one or a few points by free amino groups on the surface.

The conformational dependence of IgE binding to phospholipases was confirmed by the direct studies with RAST and RAST inhibition. Treatment of yellow jacket phospholipase similar to that encountered in preparing a sample for an SDS gel caused a 700-fold decrease in inhibitory activity.

The difference observed in IgE antibody binding between blots prepared from nondenaturing PAGE and SDS-PAGE was much less for yellow jacket and bee venoms than was observed with *S. invicta*, imported fire ant.<sup>1</sup> The SDS-PAGE gels from *S. invicta* demonstrate a much larger fraction of low-molecular-weight material, and the major bands are at lower molecular weight than the bands on the nondenaturing gels. This is consistent with either multichain structure

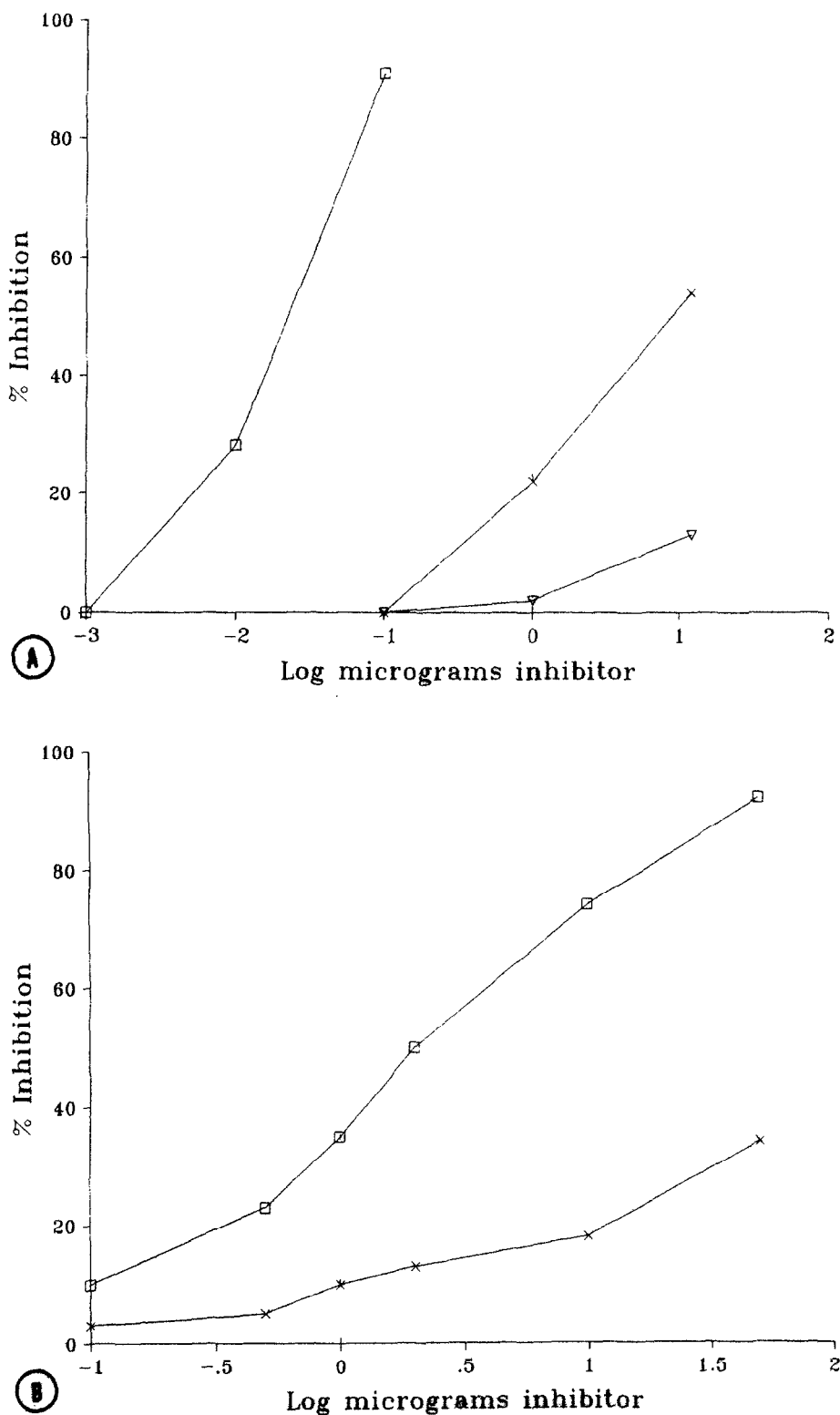


**FIG. 5.** RAST inhibition of binding to venom phospholipases. **A**, Binding to *V. maculifrons* phospholipase inhibited by native phospholipase (□), mildly reduced and alkylated phospholipase (X), and completely reduced and alkylated phospholipase (▽). **B**, Inhibition of RAST to honeybee phospholipase A by native phospholipase (□) and mildly reduced and alkylated phospholipase (X). Inhibition experiments were limited by the low solubility of reduced and alkylated phospholipases in nondenaturing buffers.

or aggregation of the venom components. In bee and yellow jacket venoms, all major allergens are composed of single polypeptide chains.<sup>2-10</sup>

Many investigators have used immunoblot techniques to investigate human IgE antibody binding to allergens. Peltre et al.<sup>17</sup> used capillary blotting from IEF gels to study grass allergens and many other systems. Similar methods have been used by other investigators to study pollen allergens<sup>18</sup> and mite allergens.<sup>19</sup> Immunoblots prepared from SDS gels, first used for detection of IgE antibodies by Sutton et al.<sup>20</sup> for studies on cereal allergens, have been used for studies of grass-pollen allergens,<sup>21</sup> and recently have been used by Dewair et al.<sup>22</sup> in an interesting study of silk allergens, and by Hussain and Ottesen<sup>23</sup> for studies of filarial allergens. None of these authors have compared immunoblot methods with purified allergen RAST or investigated the possible effects of distortion of protein tertiary structures on the results. It is probable that the dependence of IgE binding on protein tertiary structure is different for various allergens. It would not be unexpected if binding to food allergens were primarily to linear type determinants, since the molecules may be denatured by stomach acid and partially digested before encountering the IgE B-lymphocytes. By contrast, it appears that much IgE antibody produced in response to natural exposure to intact molecules (e.g., venoms injected by a sting) may be directed to topographic determinants. This would be most apparent with allergens that are composed of more than one protein chain but can also be observed with single chain molecules, such as *V. maculifrons* antigen 5 and phospholipase.

We thank Elizabeth Kopelman, Charlene Cone, and Marvin B. Alligood, Jr., for their technical assistance with this project. The live yellow jackets were collected and their venom obtained by Robert Jacobson.

## REFERENCES

- Hoffman DR. Allergens in Hymenoptera venom. XVII. Allergenic components of *Solenopsis invicta* (imported fire ant) venom. J ALLERGY CLIN IMMUNOL 1987;80:300-6.
- Hoffman DR, Golden DBK. Standardized extracts, stinging and biting insects. Clin Rev Allergy 1987;5:75.
- King TP, Sobotka AK, Alagon A, Kochoumian L, Lichtenstein LM. Protein allergens of white-faced hornet, yellow hornet, and yellow jacket venoms. Biochemistry 1978;17:5165.
- King TP, Kochoumian L, Joslyn A. Wasp venom proteins: phospholipase A<sub>1</sub> and B. Arch Biochem Biophys 1984;230:1.
- Hoffman DR, Wood CL. Allergens in Hymenoptera venom. XI. Isolation of protein allergens from *Vespula maculifrons* (yellow jacket) venom. J ALLERGY CLIN IMMUNOL 1984;74:93.
- Hoffman DR. Allergens in Hymenoptera venom. XIII: Isolation and purification of protein components from three species of vespid venoms. J ALLERGY CLIN IMMUNOL 1985;75:599.
- Hoffman DR, Shipman WH. Allergens in bee venom. I. Separation and identification of the major allergens. J ALLERGY CLIN IMMUNOL 1976;58:551.
- Hoffman DR, Shipman WH, Babin D. Allergens in bee venom. II. Two new high molecular weight allergenic specificities. J ALLERGY CLIN IMMUNOL 1977;59:147.
- Hoffman DR. Allergens in bee venom. III. Identification of allergen B as an acid phosphatase. J ALLERGY CLIN IMMUNOL 1977;59:364.
- King TP, Sobotka AK, Kochoumian L, Lichtenstein LM. Allergens of honey bee venom. Arch Biochem Biophys 1976;172:661.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. Proc Natl Acad Sci USA 1979;76:4350.
- Szewczyk B, Kozloff LM. A method for the efficient blotting of strongly basic proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose. Anal Biochem 1985;150:403.
- Hoffman DR. The use and interpretation of RAST to stinging insect venoms. Ann Allergy 1979;42:224.
- Kemeny DM, Harries MG, Youtlen LJF, MacKenzie-Mills M, Lessof MH. Antibodies to purified bee venom proteins and peptides. I. Development of a highly specific RAST for bee venom antigens and its application to bee sting allergy. J ALLERGY CLIN IMMUNOL 1983;71:505.
- Hoffman DR. Allergens in Hymenoptera venom. XIV. IgE binding activities of venom proteins from three species of vespids. J ALLERGY CLIN IMMUNOL 1985;75:606.
- Benjamin DC, Berzofsky JA, East IJ, Gurd FRN, Hannum C, Leach SJ, Margoliash E, Michael JG, Miller A, Prager EM, Reichlin M, Sercarz EE, Smith-Gill SJ, Todd PE, Wilson AC. The antigenic structure of proteins: a reappraisal. Ann Rev Immunol 1984;2:67.
- Peltre G, Lapeyre J, David B. Heterogeneity of grass-pollen allergens (*Dactylis glomerata*) recognized by IgE antibodies in human patients' sera by a new nitrocellulose immunoprint technique. Immunol Lett 1982;5:127.
- Bengtsson A, Rolfsen W, Einarsson R. Characterization of allergens and patient sera by a nitrocellulose immunoprint technique. Int Arch Allergy Appl Immunol 1985;78:139.
- Geissler W, Maasch HJ, Winter G, Wahl R. Kinetics of allergen release from house dust mite *Dermatophagoides pteronyssinus*. J ALLERGY CLIN IMMUNOL 1986;77:24.
- Sutton R, Wrigley CW, Baldo BA. Detection of IgE and IgG binding proteins after electrophoretic transfer from polyacrylamide gels. J Immunol Methods 1982;52:183.
- Ford SA, Tovey ER, Baldo BA. Identification of orchard grass (*Dactylis glomerata*) pollen allergens following electrophoretic transfer to nitrocellulose. Int Arch Allergy Appl Immunol 1985;78:15.
- Dewair M, Baur X, Ziegler K. Use of immunoblot technique for detection of human IgE and IgG antibodies to individual silk proteins. J ALLERGY CLIN IMMUNOL 1985;76:537.
- Hussain R, Ottesen EA. IgE responses in human filariasis. IV. Parallel antigen recognition by IgE and IgG4 subclass antibodies. J Immunol 1986;136:1859.