

# Studies of the antigenicity and allergenicity of phospholipase A<sub>2</sub> of bee venom

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*The antigenic and allergenic properties of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and whole bee venom were compared by measuring the IgG and IgE antibody responses in animals and man. Precipitating antibodies raised in rabbits and reaginic and other antibodies raised in mice reacted about equally with both bee venom and PLA. The majority of human sera containing bee venom-specific IgE also contained PLA-specific IgE, although in somewhat lower titers. Similarly, most human sera with significant amounts of total antibodies reacting with bee venom also had antibodies reacting with PLA. Histamine and SRS-a release from leukocytes of sensitive patients followed challenge with whole bee venom and PLA in the majority of instances. However, mediator release from several patients' cells was obtained with bee venom only. These studies suggest that although PLA is a major allergen and antigen in bee venom, significant exceptions in patients' reactivity may limit its potential diagnostic and therapeutic usefulness.*

Bee venom is a complex mixture of enzymes and proteins. At least nine major fractions have been isolated from bee venom.<sup>1, 2</sup> Of these, phospholipase A<sub>2</sub> (PLA) has been identified as a major allergen.<sup>3</sup> This association has practical ramifications including the diagnostic and therapeutic use of PLA for individuals with bee allergy, and the use of PLA assays as a means of standardization of bee venom and bee body extracts. It therefore seemed important to study the antigenicity and allergenicity of phospholipase A as compared to whole bee venom.

## MATERIALS AND METHODS

### Source of materials

Bee venom was obtained from the Sigma Chemical Co., St. Louis, Mo., or locally by electrical stimulation according to the method of Benton.<sup>4</sup> This latter source will be indicated by

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Supported in part by United States Public Health Service Allergic Diseases Clinical Centers Grant 2-P15-AI-10397 and in part by United States Public Health Service Research Grant 5-RO1-AI-01303 of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Presented in part at the Thirty-first Annual Meeting of the American Academy of Allergy, San Diego, Calif., February, 1975.

Received for publication Sept. 18, 1975.

Accepted for publication Jan. 21, 1976.

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\*Recipient of the Dr. Henry C. and Bertha H. Buswell Research Fellowship.

an asterisk. PLA was obtained from the Sigma Chemical Co. or from Dr. Willard Elliot.<sup>1</sup> The activity of the two PLA preparations appeared to be equal.

### Animal models

*Rabbit antibodies.* Rabbits were immunized with 50  $\mu$ g of bee venom\* or 11  $\mu$ g of PLA in 0.5 ml of complete Freund's adjuvant. After the initial immunization, booster injections were given at 3 and 6 wk. Sera were obtained and analyzed for the presence of precipitating antibodies to bee venom and PLA by gel diffusion techniques.

*Reaginic antibodies in mice.* A/HeJ mice were immunized with bee venom\* and PLA by the method described by Levine and Chang<sup>5</sup> and Charavejasarn, Reisman, and Arbesman.<sup>6</sup> Reaginic antibodies were measured by the 48-hr passive cutaneous anaphylaxis (PCA) method. In addition, gamma<sub>2</sub> antibodies detected by the 2-hr PCA test and hemagglutinating antibodies were also measured.

### Human studies

Sera, and in some instances cells, were obtained from (1) patients who had typical immediate allergic reactions following bee stings, (2) beekeepers who had very little local reaction following bee stings and who represent an "immune" population, and, (3) normal, nonallergic individuals used as controls. The following procedures were carried out.

*Radioallergosorbent test (RAST).* Bee venom-specific IgE antibodies and PLA-specific IgE antibodies were determined by the RAST using a modification of the technique described by Ceska, Eriksson, and Varga.<sup>7</sup> Twenty milligrams of bee venom or 5 mg of PLA were coupled to 200 cyanogen bromide-activated cellulose discs. To each disc, 0.05 ml of the patient's serum was added. The samples were allowed to incubate at room temperature for 3 hr. After washing 4 times with normal saline, pH 7.40, 0.05 ml of radioactive antihuman IgE<sup>8</sup> was added to each tube and the samples were incubated overnight at room temperature. The following day after further washings the supernatant was removed and the tubes were counted in a Packard Auto-spectro gamma counter.

RAST results were expressed in relationship to a known ragweed pooled sera standard which was assayed in each experiment. This standard was obtained by pooling serum from several ragweed-sensitive individuals. The ragweed IgE antibody concentration of this pool was measured by a radioimmunoassay<sup>9</sup> and was approximately 133 ng IgE/ml. The ragweed sera standard was incubated with cellulose discs prepared by coupling 10 ml of 15% ragweed extract in 10 ml of 0.1 M NaHCO<sub>3</sub> per 200 discs by the same method described for bee venom. By using such a standard in each experiment, the amount of a fixed quantity of IgE antibody in the standard could be compared in a relative manner to an unknown quantity of specific IgE antibody by comparison of the counts per minute (cpm) of each sample. This is a similar approach to that used by Johansson, Bennich, and Berg.<sup>10</sup>

Calculations were performed as follows:

$$\frac{\text{Net cpm bee venom}}{\text{Net cpm ragweed standard}} = \frac{X}{133\%}$$

Thus, if the net cpm for the bee venom sample was 8,000, and the net cpm of the ragweed standard 10,000, then  $X = 106\%$ .

All samples were run in duplicate. The standard discs were run in quadruplicate. The disc RAST had an average standard deviation equal to 7% of the mean cpm. Twenty mg of bee venom and 5 mg of PLA per 200 discs appeared to represent the capacity of the discs for binding of these antigens. Higher concentrations of antigen did not increase RAST values.

*RAST inhibition studies.* Sera from 6 bee-sensitive individuals were pooled, 0.15 ml of serial 10-fold dilutions of either bee venom or PLA was added to 0.05 ml of pooled sera. Two

\*Asterisk used throughout to indicate source of bee venom from Sigma Chemical Co., St. Louis, Mo.

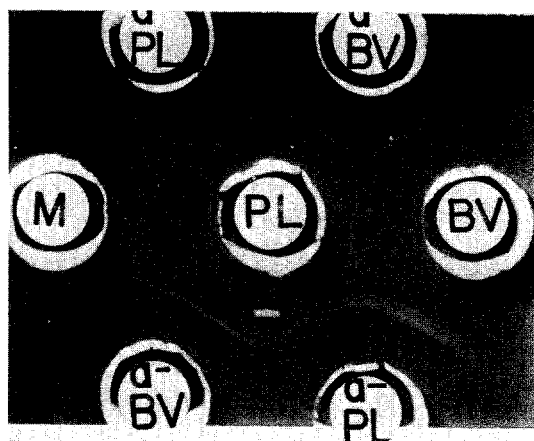


**FIG. 1.** Double diffusion gel precipitation experiment. Rabbit antisera to PLA ( $\alpha$ -PL) and bee venom ( $\alpha$ -BV) are in the upper and lower wells. Mellitin (M), PLA (100  $\mu$ g/ml, PL), and bee venom (100  $\mu$ g/ml, BV) are in the middle wells. A line of identity is present between bee venom and PLA and their antisera. Mellitin is a major component of bee venom.

percent human serum albumin (HSA) was used in place of antigens in one set of tubes. All samples were set up in duplicate. After overnight incubation at 4° C, the cellulose discs coated with either bee venom or PLA were added to each tube and the procedure continued as outlined above. The cpm obtained for the tubes incubated with 2% HSA were assigned the value of 100%. The percent of inhibition achieved by each antigen was based on the following equation:

$$\% \text{ inhibition} = \left( 1 - \frac{\text{net cpm sample}}{\text{net cpm without antigen}} \right) \times 100.$$

#### *Mediator release from leukocytes.*

**HISTAMINE.** The modification of May and colleagues<sup>11</sup> of the Lichtenstein and Osler method<sup>12</sup> was used for this assay. Leukocytes were obtained from 11 insect-sensitive individuals. Histamine release was induced by challenge with 2.5 to 0.0006  $\mu$ g/ml bee venom or PLA.

**SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS-A).** The method described by Brocklehurst<sup>13</sup> was employed to assay for the presence of SRS-a. This assay utilized guinea pig ileum in a 10-ml bath. The standard for this assay, kindly supplied by Dr. Robert Orange, was prepared from rat peritoneal mast cells. One unit of this standard caused a 20-mm contraction of guinea pig ileum; 17.5 mg of the dry standard was dissolved in 1 ml of Tyrode solution. A 1:250 dilution was prepared. When 0.4 ml of this diluted standard was added to the 10-ml bath, a 20-mm contraction was produced. SRS-a release from patient leukocytes was measured after incubating bee venom or PLA in a final concentration of 10  $\mu$ g/ml with 1 ml of patient leukocytes for 45 min at 37.0° C. The tubes were centrifuged, the supernatant was added to the test bath, and the contraction of the guinea pig ileum was recorded.

**Measurement of total antibodies.** Total antibodies reacting with bee venom and PLA were determined by a competitive radioimmunoassay developed in this laboratory.<sup>9</sup> This assay measures antibodies of all immunoglobulin classes. Previous studies have shown high levels of total antibody in the sera of beekeepers and one individual treated with bee venom. Sera taken from normal control individuals had low levels of total antibodies.<sup>14</sup>

## **RESULTS**

### **Animal models**

Rabbits immunized with bee venom and PLA developed precipitating antibodies demonstrable by gel diffusion. Lines of identity between bee venom and

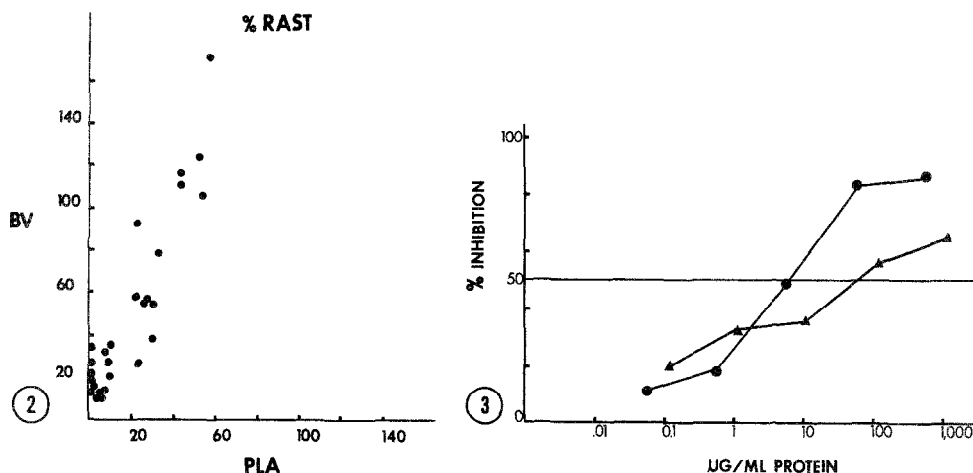


**FIG. 2.** The relationship between bee venom and PLA-specific IgE antibodies in sera of 25 untreated insect-allergic patients.

**FIG. 3.** Inhibition of the bee venom RAST by bee venom (●—●) and PLA (▲—▲). Six  $\mu\text{g/ml}$  of protein of bee venom and 55  $\mu\text{g/ml}$  of protein of PLA were required for 50% inhibition.

PLA were found with antisera raised against both antigens. A typical example is shown in Fig. 1.

Reaginic antibodies measured by 48-hr PCA were raised in mice by immunization with bee venom and PLA. Both reaginic antibodies reacted to both antigens, i.e., antibee venom reagins reacted with PLA and vice versa.

Antibodies detected by the 2-hr PCA (IgG<sub>1</sub>) and precipitating antibodies were also present in mice following immunization with both antigens. These antibodies also reacted to both bee venom and PLA.

### Human studies

*RAST measurement of specific IgE antibodies.* From a group of 212 patients with histories of insect sensitivity, sera from 65 patients were found to contain significant amounts of IgE antibodies to bee venom as defined by levels greater than 10%. Of these 65 sera, 54 had levels of 5% or greater PLA-specific IgE and 37 had levels of 10% or greater PLA-specific IgE. There were no instances of antibodies detected against PLA in the absence of reactions with bee venom.

There was a very good correlation of the relative quantitative amounts of antibody reacting with PLA and bee venom. Twenty-five sera of untreated bee-sensitive patients were assayed simultaneously for bee venom and PLA-specific IgE. Mean antibody values of 47% for bee venom and 19% for PLA were found. These values are plotted in Fig. 2. There is a positive correlation between these values ( $r = 0.805$ ,  $p < 0.001$ ).

### RAST inhibition studies

Inhibition of the bee venom RAST by bee venom and PLA is shown in Fig. 3. Fifty percent RAST inhibition was accomplished by 6  $\mu\text{g/ml}$  protein of bee venom and 55  $\mu\text{g/ml}$  protein of PLA.

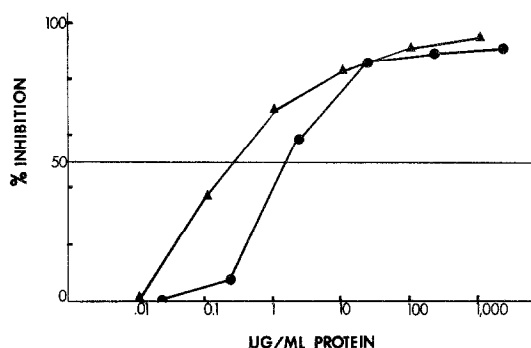


**FIG. 4.** Inhibition of the PLA RAST by bee venom (●—●) and PLA (▲—▲); 1.7  $\mu\text{g/ml}$  of protein of bee venom and 0.25  $\mu\text{g/ml}$  of protein of PLA were required for 50% inhibition.

**TABLE I.** Histamine and SRS-a release from cells of bee-sensitive patients following challenge with bee venom (BV) and PLA

| Pt.   | % histamine released with 0.625 $\mu\text{g/ml}$ |     | SRS-a release (U) |     |
|-------|--------------------------------------------------|-----|-------------------|-----|
|       | BV                                               | PLA | BV                | PLA |
| D. C. | 95                                               | 98  | 2.9               | 1.6 |
| T. E. | 89                                               | 75  | 3.5               | 2.6 |
| S. E. | 78                                               | 67  | ND                | ND  |
| S. M. | 55                                               | 61  | 1.0               | 0   |
| L. D. | 52                                               | 11  | 3.0               | ND  |
| S.    | 45                                               | 0   | 2.0               | 0   |
| T. G. | 38                                               | 34  | 0.8               | 0   |
| G. T. | 24                                               | 5   | 1.8               | 0.9 |
| J. M. | 21                                               | 4   | 1.3               | 0.6 |
| J. S. | 6                                                | 21  | 2.9               | ND  |
| M. S. | 4                                                | 4   | 1.0               | ND  |

Inhibition of the PLA RAST was also done. For this experiment a different pool of sera obtained from 3 patients known to have PLA-specific IgE in levels greater than 30% was used. The inhibition data are shown in Fig. 4. Fifty percent RAST inhibition was accomplished with 0.25  $\mu\text{g/ml}$  of PLA and 1.7  $\mu\text{g/ml}$  of bee venom. Almost total inhibition occurred with large amounts of both antigens.

#### Mediator release from leukocytes

**Histamine.** The percent histamine release obtained after challenge with 0.625  $\mu\text{g/ml}$  of either bee venom or PLA was compared for the 11 patients. The results are reported in Table I along with corresponding values for SRS-a release. After bee venom challenge, leukocytes released from 4% to 95% histamine (mean, 46%). The corresponding concentration of PLA released from 0% to 98% histamine (mean, 35%). At this concentration of antigen, 3 patients showed significant histamine release with bee venom and no release with PLA. These data are plotted in Fig. 5. Control cells from 14 patients released from 0% to 27% histamine (mean, 4%) after challenge with 0.625  $\mu\text{g/ml}$  bee venom. Control cells from 10 patients released from 0% to 7% histamine (mean, 1%) after challenge with an identical concentration of PLA.

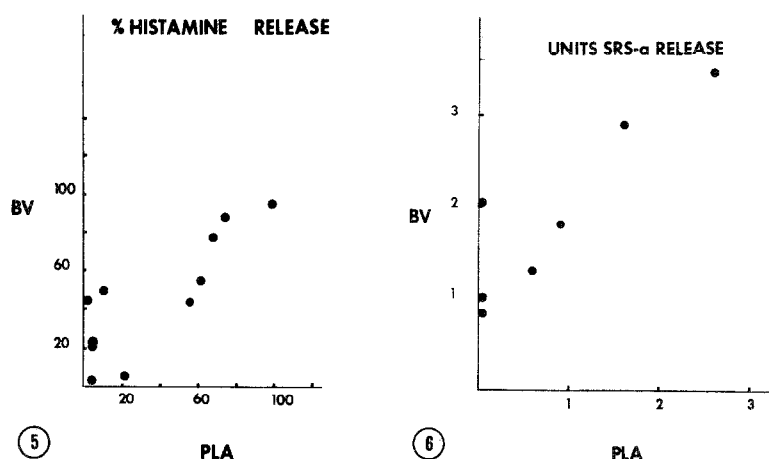


**FIG. 5.** Histamine release from cells of 11 bee-sensitive patients induced by bee venom and PLA.

**FIG. 6.** Release of SRS-a by challenge with bee venom plotted against release of SRS-a by challenge with PLA.

**TABLE II.** The presence of total antibodies to bee venom (BV) and PLA in beekeepers, untreated patients, and normal control patients

|                             | N  | BV               | PLA             |
|-----------------------------|----|------------------|-----------------|
| Beekeepers                  | 25 | 90.5* (0.03-500) | 83.8* (0.1-500) |
| Untreated allergic patients | 17 | 51.2 (0.01-210)  | 17.8 (0-87.5)   |
| Normals                     | 51 | 1.4 (0-18)       | 1.2 (0-12)      |

\* $\mu\text{g/ml}$ .

*SRS-a.* Seven patients had SRS-a release assayed after incubation with 10  $\mu\text{g/ml}$  of bee venom or PLA. These results are plotted in Fig. 6. Four patients released significant amounts of SRS-a in response to both antigens. Three patients released SRS-a only after bee venom challenge. Cells from 10 control individuals were assayed for SRS-a after incubation with an equal concentration of bee venom. From 0 to 0.96 U were released (mean, 0.1 U). After incubation with PLA, cells from 8 control individuals released from 0 to 0.5 U (mean, 0.05 U).

### Total antibodies

Total antibodies reacting with bee venom and PLA were measured in the serum of 18 untreated insect-sensitive individuals, 51 normal controls, and 25 beekeepers. The results are shown in Table II. Total antibodies were highest in beekeepers. Insect-sensitive individuals had higher values than those found in the normal population. The absolute levels were higher with bee venom than with PLA. Generally, the pattern of reactivity was the same for both antigens.

### DISCUSSION

The results of these studies measuring both the IgE and IgG responses and mediator release suggest that PLA is a major antigen in bee venom. However,

there appear to be a small minority of patients who do not have antibodies directed at PLA.

In the rabbit studies, antibodies induced by immunization with bee venom and PLA showed evidence of identity in gel diffusion experiments. No lines of precipitation between either antigen and normal rabbit sera were found. These latter findings differ from those of Franklin and Baer,<sup>15</sup> who found nonimmune precipitation bands between bee venom and PLA and normal rabbit sera. The concentrations of antigen used in their experiments were considerably greater than in ours, and may explain the different results.

The reaginic antibodies raised in mice as measured by the 48-hr PCA reaction showed evidence of cross-reactivity with bee venom and PLA.

In the human studies, specific IgE antibodies measured by the RAST procedure and by histamine release from leukocytes generally reacted with both bee venom and PLA. There was a quantitative relationship in the degree of reactivity. All patients reacting to PLA by RAST and histamine release also reacted with bee venom.

Significantly, there were patients who had IgE antibodies reacting with bee venom who did not show similar reactions with PLA. Approximately 15% of the sera assayed by RAST did not react with PLA. Similarly, 3 patients were identified who showed significant histamine release with bee venom and failed to react to PLA at a concentration of antigen considered significant.

SRS-a has been identified as an important chemical mediator released from mast cells and basophils. Of the 7 patients studied who released SRS-a after bee venom challenge, only 4 reacted to PLA.

The presence of IgG antibodies in humans was assayed indirectly by the total antibody measurement. This technique actually measures antibodies associated with all immunoglobulin classes. The antibody titers reacting with bee venom were generally higher than found with PLA in all three groups of patients tested: insect-allergic patients, controls, and beekeepers. There were some quantitative relationships between these two antigens, however. Sera with increased titers against bee venom showed increased titers with PLA.

These data suggest that most patients reacting to bee venom either with IgE or IgG antibodies show reactivity to PLA. It is our impression that PLA is indeed a major antigen in bee venom. It is important, however, to point out that there are definite exceptions. The other components of bee venom that may be antigenic are presently being investigated. These results suggest that whole bee venom is a good material for diagnostic testing and that PLA cannot be relied upon as an adequate substitute.

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