Allergy to insect stings

II. Phospholipase A: The major allergen in honeybee venom

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In order to determine the proteins of major allergenic importance in honeybee venom (Apis mellifera) it was chromatographed on G-50 Sephadex. The four major protein peaks eluted were identified as hyaluronidase, phospholipase, melittin, and apamin. Testing these preparations on the leukocytes of 6 honeybee-sensitive patients, with the in vitro method of histamine release, revealed that all individuals were most sensitive to phospholipase A. IgE antibodies against phospholipase A (RAST) were found in the sera of honeybee-sensitive patients and IgG antibodies to this venom component were found in the sera from beekeepers and venom-treated patients. Melittin appeared to be allergenic in several patients, but the results were variable and were possibly due to contamination with phospholipase. All patients were insensitive to the hyaluronidase and apamin preparations. We conclude that phospholipase A is the major allergen of honeybee venom and, since this protein is readily available, it should be useful for diagnostic and therapeutic studies as well as for the standardization of materials used in the management of honeybee-sensitive patients.

The study of Hymenoptera sensitivity has been severely hampered by the use of impure and largely irrelevant extracts prepared from the whole ground bodies of these insects. Because of their widespread use, specific diagnosis was impossible¹,² and even the "allergic" pathogenesis of the disease was legitimately called into question. Although some voices were raised against this practice, notably that of Loveless,³ this state of affairs has persisted for the last 30 years. We and others have recently used more modern tools to reinvestigate this problem and have been able to clearly demonstrate the utility of venoms in the diagnosis of Hymenoptera sensitivity.⁴ It has, moreover, been possible to clearly implicate the IgE antibodies as the pathogenetic mechanism; preliminary therapeutic experience appears to confirm the importance of venoms.⁶,⁷ It is, of course, recognized that the venoms themselves contain multiple proteins

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and therefore several potential allergens. To further assist diagnosis it is important to identify them; it is also imperative to have relatively clean allergens to use the RAST technique effectively and the same requirement exists for IgG antibody measurements. We have begun this work with the best characterized venom, that of the honeybee (*Apis mellifera*), in an effort to determine the major venom allergen and to obtain a preparation pure enough to carry out the in vitro diagnostic tests cited.

Early studies of insect venoms by Jaques and Schacter\(^9\) revealed the presence of various vasoactive amines and kinins in wasp venom. Following the work of Benson and Semenov,\(^9\) who found a beekeeper sensitive to both bee venom and whole insect extract, O'Connor, Rosenbrook, and Erickson\(^10\) and Shulman, Langlois, Arbesman, and their associates\(^11a, b, c, d\) compared the antigenic content of whole insect bodies with that of venom sac preparations. Both groups found several antigens in the venoms not present in sacless bodies. Many investigators have studied the protein content of various pure venoms, especially that from the honeybee. The classic work of Habermann\(^12\) led to the isolation and characterization of many of the components of the venoms of several Hymenoptera classes. In the case of the honeybee, hyaluronidase, melittin, phospholipase, and apamin make up greater than 70% of the venom protein. Several honeybee venom proteins have been well characterized and Shipolini and associates\(^13a, b\) have determined the primary amino acid sequence of the phospholipase A. Melittin has also been sequenced\(^14\) and then synthesized by investigators in Germany.\(^15\) This work has left unanswered, however, the question of which proteins in the venom were responsible for inducing the allergic response. The present study is directed to that question. With the use of simple chromatographic techniques and the leukocyte-histamine release model of "in vitro anaphylaxis," our results indicated that phospholipase A is the major allergen in honeybee venom.

**METHODS**

**Antigens**

The antigens used for these studies were honeybee venom, commercial phospholipase A (Sigma Chemical Company, Saint Louis, Mo.) and 4 pools obtained from commercial honeybee venom by Sephadex G50 chromatography. The honeybee venom used was collected both by electrical stimulation and by allowing the insects to sting through a cellophane membrane.\(^16\) The dried venom was weighed in milligram quantities, diluted in Tris-buffered saline, dialyzed, and stored at \(-20^\circ\) C as previously described.\(^4\) Phospholipase A from bee venom was treated similarly except that dialysis, used to remove substances from whole bee venom that interfere with the fluorometric assay for histamine, was not necessary. Separation of honeybee venom components was carried out as previously described.\(^17\) A Sephadex G50 fine (Pharmacia, Piscataway, N. J.) gel column was prepared and calibrated with blue dextran 2000 (Pharmacia) and with chymotrypsinogen, cytochrome C, and insulin as molecular weight markers. Then 500 mg of bee venom were placed on the column, the eluates were collected at a constant volume of 4.8 ml, and the protein content of each tube was determined at an O.D. of 280 nM. Alternatively (see below) the Folin-Ciocalteau reaction was used to estimate protein. Chromatography yielded 4 major protein peaks. Portions of the individual tubes comprising each peak were pooled, equalized as to protein content, aliquoted, and stored at \(-20^\circ\) C until assayed. The individual eluates were also aliquoted and frozen until used.
Previously described functional studies identified three pools as being largely composed of hyaluronidase (Pool I), phospholipase A (Pool II), and melittin (Pool III). Based on Habermann's data, Pool IV was assumed to contain apamin.

Antisera

Antiserum to phospholipase A was prepared in two New Zealand white rabbits (Bunnyville Farms, R.D. 2, Littlestown, Pa.) by injecting 500 μg/ml in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) into both rear footpads. Two weeks later, the rabbits received an intramuscular injection of 500 μg of phospholipase A in saline and were bled 2 weeks later. The antiserum used in our in vitro studies was a pool made from this final bleeding; on immunodiffusion a single line of identity between purified phospholipase A and unfractionated honeybee venom was observed. Rabbit anti-whole bee venom was prepared according to a similar regimen; a total dose of 11 mg of venom was injected into each rabbit and the pool was made from several bleedings.

Human antisera from 10 beekeepers was generously provided by Dr. A. W. Benton; sera from venom-treated patients were provided by Dr. Charles Reed and from our own practice. Drs. Halla Brown and Solomon Barr provided sera from patients on long-term therapy with whole-body insect extracts. Other sera from similar patients were obtained in our Allergy Clinic.

Histamine release studies

Patient sensitivity to honeybee venom, phospholipase A, and bee venom components was assessed with the leukocytes of known honeybee-allergic patients and normal control subjects as previously described. Patient sensitivity to honeybee venom, phospholipase A, and bee venom components was assessed with the leukocytes of known honeybee-allergic patients and normal control subjects as previously described. Briefly, leukocytes were collected by dextran sedimentation, washed, resuspended in TACM (Tris-buffered saline containing calcium, magnesium, and human albumin), and mixed with appropriate concentrations of each antigen, usually covering a range of 10⁻⁵-10⁻⁶ μg of protein per milliliter. After 60 min at 37°C the histamine release was determined fluorometrically. For passive sensitization experiments the recipient cells, previously shown to be nonreactive to honeybee venom, were sensitized following the techniques described by Levy and Osler.

Antibody studies

Assessment of serum blocking antibodies using histamine release was carried out as previously described: appropriate dilutions of serum were incubated with the antigens for 30 min at 25°C prior to the addition of leukocytes and the amount of histamine release in these tubes compared with those in which antigen was incubated with control serum.

Specific IgG anti-phospholipase A antibodies were measured with a double-antibody method based on the Farr technique with modifications including those described previously. Specific ¹²⁵I-labeled phospholipase A was mixed with the serum to be tested, allowed to react for 4 hr at 25°C, and then the serum IgG precipitated with a specific goat anti-human γ chain. After 18 hr at 4°C the precipitate was washed free of unbound antigen and the specific ¹²⁵I-phospholipase A determined.

Specific IgE antibodies were measured with the RAST technique described by Wide, Bennich, and Johansson with few modifications.

RESULTS

Gel filtration chromatography of honeybee venom yielded 4 protein peaks, with molecular weights of approximately 30,000, 23,000, 4,000, and 1,500, respectively. A typical elution pattern is shown in Fig. 1. A fifth peak, seen at about tube 100, was not further studied since the low molecular weight substances, including histamine, which it contains are of little interest as allergens. Gel diffusion analysis of the first 80 tubes with a rabbit antiserum to whole bee
venom revealed the presence of an antigen between tubes 30 and 36, and a second in tubes 38 through 48, which overlapped into tubes 50 through 63. Elution tubes beyond the sixty-fifth showed only nonimmune precipitation as ascertained when normal rabbit serum was used as a control.

With the use of information obtained from protein determinations and from the antigenic patterns, aliquots of the samples in each tube comprising the peaks were pooled. As noted above, they were largely made up of (I) hyaluronidase, (II) phospholipase A, (III) melittin, and (IV) apamin. These 4 pools were not further purified for this study; each probably contains trace amounts of the adjacent protein(s).

The leukocytes of 6 patients with clear-cut clinical and laboratory sensitivity to honeybee venom were challenged in vitro with unfractionated honeybee venom and each of the four chromatographic fractions. The results of two typical experiments are shown in Fig. 2. The leukocytes of both patients were most sensitive to Pool II containing phospholipase A; 50% histamine release occurred at $3 \times 10^{-5}$ and $5 \times 10^{-4}$ µg protein/ml, respectively. The same response to unfractioned venom required 3- to 10-fold more protein and challenge with Pools I, III, and IV showed that 100- to 1,000-fold more protein was required to elicit 50% histamine release. Some patients were insensitive to one or more venom components (e.g., Pool I, Fig. 2, upper panel). Similar patterns of release were found with the leukocytes of the 6 allergic patients tested, whereas the response of 6 normal control subjects to bee venom or to the protein pools was uniformly negative (Table I).

Since Pool II appeared to be the most active we explored its homogeneity by studying aliquots of the individual elution tubes (38-56) for their ability to release histamine from sensitive leukocytes. Each tube was equalized as to protein content and tested over a concentration range from $10^{-8}$ to 10 µg/ml. The results of experiments with 2 allergic patients are shown in Fig. 3. Two peaks of allergenic activity were found; one occurred in tube 42, the second in tubes 52 to 54.
TABLE I. Summary of histamine release data using bee venom and bee venom components

<table>
<thead>
<tr>
<th>Patient</th>
<th>Honeybee venom Protein (ng/ml) for 50% histamine release</th>
<th>Phospholipase A Sigma Pool I</th>
<th>Pool II</th>
<th>Pool III</th>
<th>Pool IV</th>
<th>Yellow jacket venom</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. M.</td>
<td>2.0</td>
<td>0.3</td>
<td>150</td>
<td>1.0</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>P. P.*</td>
<td>1.0</td>
<td>1.0</td>
<td>1,000</td>
<td>0.5</td>
<td>40</td>
<td>3,000</td>
</tr>
<tr>
<td>R. W.</td>
<td>2.0</td>
<td>&gt;1,000</td>
<td>0.5</td>
<td>200</td>
<td>ND</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>N. M.</td>
<td>3.0</td>
<td>2.0</td>
<td>1,000</td>
<td>2.0</td>
<td>3.0</td>
<td>ND</td>
</tr>
<tr>
<td>K. B.*</td>
<td>1.0</td>
<td>0.1</td>
<td>ND</td>
<td>1.0</td>
<td>20</td>
<td>1,200</td>
</tr>
<tr>
<td>J. V.</td>
<td>0.1</td>
<td>0.04</td>
<td>&gt;100</td>
<td>0.05</td>
<td>5.0</td>
<td>50</td>
</tr>
<tr>
<td>N. G.†</td>
<td>3,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>E. G.‡</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

*Passive sensitization.  
†Vespid allergic.  
‡Nonallergic control.

These peaks bear little relation to the protein content but rather correspond to the ascending and descending limbs of the Pool II peak. Essentially the same protein concentration (∼ 3 × 10⁻⁴ μg) was required to elicit a 50% response when either tube 42 or 52 was tested; when Pool II itself was studied on the leukocytes of the same patients, 5 × 10⁻⁴ μg protein/ml was sufficient to elicit 50% histamine release.

Commercially purified phospholipase A is readily obtained and was compared for potency with Pool II and the unfractionated venom. Two such assays are shown in Fig. 4. The concentrations of Pool II and of purified phospholipase A required for 50% histamine release were essentially the same; both patients were more sensitive to this venom component than to the unfractionated venom. Also shown are the results of exposing the cells of normal donors to phospholipase A; they were completely negative here as they were in 6 other experiments, in which concentrations of phospholipase A up to 10 μg/ml were used.

Histamine release data obtained with the use of unfractionated venom, the 4 venom pools, and commercial phospholipase A are summarized in Table I. Results obtained with yellow jacket venom are included for comparison. All 6 honeybee allergic patients reacted to nanogram amounts of honeybee venom, commercial phospholipase A, and Pool II. With the exception of one passive sensitization experiment (P. P.), all patients were more sensitive to phospholipase A and Pool II than to unfractionated venom. Their sensitivity to the other 3 pools was variable; most patients required much higher protein concentrations for histamine release. Pool I containing hyaluronidase and Pool IV containing apamin caused histamine release from sensitive leukocytes but at protein concentrations ∼ 1,000-fold greater than that required to elicit a response when Pool II was used. Pool III containing melittin appeared to be of significance as an antigen in several patients studied. Patients N. M. and J. V. both reacted to nanogram amounts of Pool III. It is possible, however, that Patient J. V. was responding to a 1% contamination of phospholipase A present in Pool III. Moreover, the protein concentration of Pool III required for histamine release varied widely and in all but one patient (N. M.) a 50- to
FIG. 2. Two experiments comparing histamine release elicited by unfractionated venom to that released with the 4 protein pools from honeybee venom.

1,000-fold increase in protein over that required with Pool II was necessary to cause significant (≥ 50%) release. Pool III was tested without effect on the leukocytes of control patients at concentrations up to 10 μg/ml to rule out the nonspecific histamine-releasing activity of melittin. This occurs only at concentrations ≥ 100 μg/ml. Further studies are necessary to determine whether the activity found in Pool III was due to melittin or to contamination of this pool with phospholipase A.

**Antibody studies**

If phospholipase A is the major allergen in honeybee venom, IgE antibodies against this antigen should be readily detectable. The sera of all 6 patients evaluated in this study were found to contain such antibodies. Three titration curves and appropriate controls are shown in Fig. 5. Control sera from patients sensitive to other species of Hymenoptera and from nonallergic controls did not contain measurable IgE antibodies to phospholipase A.

Similarly, if phospholipase A is the major allergen of honeybee venom, specific antibodies against this material should markedly decrease the activity of whole bee venom. Anti-phospholipase A markedly inhibited histamine release.
Allergy to insect stings. II

FIG. 3. Histamine release from the leukocytes of 2 patients sensitive to honeybee venom with protein from the individual tubes comprising Pool II (black triangles and circles). The protein peak, visualized at O.D. 280 nM, is shown by the open circles.

induced by unfractionated venom, Pool II, and phospholipase A. The results of 2 such experiments are shown in Fig. 6. A 1/1000 dilution of the rabbit serum caused >80% inhibition of antigenic histamine release from the cells of both patients. In both cases, a lower concentration of the monospecific antisera was required to cause 50% inhibition of phospholipase A–induced histamine release than that necessary when the antigen was unfractionated venom.

These results led us to determine whether IgG anti-phospholipase A antibodies were produced in human subjects as a result of bee stings. Consequently, we studied the sera of 10 multiply stung beekeepers and from two patients treated with whole bee venom. Antibodies of the IgG class able to bind 125I-labeled phospholipase A were found in all of the sera studied. Sera from control patients who could not recall having ever been stung lacked measurable IgG antibodies against phospholipase A, as did the sera of 5 vespid-allergic patients. The results from a typical experiment are shown in Fig. 7. It is of interest that at a 1/1000 dilution, sera from beekeepers and from venom-treated patients bind similar amounts of phospholipase A. Sera from honeybee–allergic patients, from patients treated with mixed insect extracts, and from some nonallergic control subjects who had recently been stung also bound measurable amounts of the labeled phospholipase A; however, all such sera contained 100–1,000-fold
FIG. 4. Two experiments comparing histamine release from honeybee-sensitive leukocytes using unfractionated honeybee venom, commercial phospholipase A, and Pool II as the antigens. Note the lack of histamine release with the use of phospholipase A on normal control cells.

FIG. 5. Measurement of IgE anti-phospholipase A antibodies in honeybee-allergic, vespid-allergic, and normal sera. Note the low level of antibody in all control sera.

less antibody than that from persons repeatedly injected with venom. These data will be presented in detail in a subsequent report. Sera from the beekeepers and the two venom-treated patients were also found to significantly inhibit both venom- and phospholipase-induced histamine release (data not shown). In most experiments, complete inhibition of histamine release was obtained with a 1/1000 dilution of serum.

DISCUSSION

This report suggests that phospholipase A is the major allergen in honeybee venom. All allergic patients responded to this venom protein at concentrations
FIG. 6. Inhibition of histamine release from bee-sensitive leukocytes by rabbit anti-
phospholipase A. The antibody is effective in inhibiting honeybee venom, commercial
phospholipase A, and Pool II.

FIG. 7. IgG anti-phospholipase A in sera from multiply stung and venom-treated patients.
Note the lack of specific antibodies in control sera.

equal to or less than that required to elicit histamine release with the unfrac-
tionated venom, while showing little sensitivity to the other venom proteins.
The 6 patients were selected because they were found to be sensitive to honeybee
venom and not to venoms from other Hymenoptera classes as judged by labora-
tory and clinical criteria. Control patients and patients sensitive to other
Hymenoptera species were insensitive to bee venom and to its protein com-
ponents. IgE antibodies specific for phospholipase A were also found in the
sera of honeybee–allergic patients. Reisman and associates have data in support
of this observation but also report a small number of patients whose serum
contained IgE antibodies to unfractionated venom although no detectable
antibodies to phospholipase A could be found. This discrepancy is difficult to explain. It is to be noted, however, that the RAST test is difficult to interpret when the antigen used is not clean; indeed this is one of the reasons this study was initiated.

The presence of specific IgG antibodies against phospholipase A in the sera of multiply stung persons also substantiates the importance of this venom component as a major antigen, as do our results with a monospecific phospholipase A antisera. Although, as expected, less antiserum was required to inhibit phospholipase A histamine release, inhibition of venom-induced release required only a small increase in antiserum concentration.

This is a preliminary study. A more definitive investigation requires that each protein be isolated by multiple chromatographic steps until greater than 99% homogeneity can be demonstrated. Such studies are under way. Their importance is attested to by the clear demonstration of multiple allergenic forms of phospholipase A in Pool II. It has previously been reported that phospholipase A exists in several molecular species differing in molecular weight, probably on the basis of carbohydrate differences. Two peaks of antigenic activity were found in Pool II that differed in size but not in antigenic or allergenic activity. Most major allergens, such as ragweed antigen E and rye grass Group I, exist in nature as a series of isoallergens of slightly different composition but with a complete antigenic and allergenic cross-reactivity. It appears that phospholipase A shares this characteristic.

We feel that the establishment of the major allergenic component in bee venom is of importance as a diagnostic tool which can be rapidly exploited. Phospholipase A is readily available commercially in large quantities and could be used diagnostically in the RAST test or in skin testing. The ability to predict sensitivity to honeybees in the majority of patients by means of the RAST technique will serve to alleviate many of the diagnostic problems facing physicians and perhaps to prevent unnecessary treatment with irrelevant antigens.

The same comments relate to the utility of phospholipase A in assessing the titer of IgG antibodies in monitoring the response to treatment. Recent reports from our laboratory and from Busse and associates support the concept of a role for IgG anti-phospholipase A antibodies in protection against anaphylaxis. Both venom-treated patients reached IgG anti-phospholipase A antibody titers equal to those found in the sera of beekeepers and were subsequently stung without reaction.

Finally, the establishment of phospholipase A as the major allergen of honeybee venom is important for standardization. It has been shown that the potency of whole pollen extracts, such as that from grass or ragweed pollens, can be more meaningfully evaluated when the standardization is based on the content of a known major allergen than when it is judged with protein nitrogen units or per cent solids as criteria. The phospholipase A content can be used to standardize and quantitate honeybee venom and, if necessary, to assess whether the whole body extracts have any venom proteins.
NOTE ADDED IN PROOF

Our recent studies have revealed that hyaluronidase (Peak I) is rapidly denatured after isolation. If this is prevented, hyaluronidase is antigenic in most patients (King et al.: Allergens of honeybee venom. In press, Arch. Biochem. Biophys.) However, all honeybee-allergic patients are highly sensitive to Phos A as judged by several immunologic parameters.

REFERENCES