

Melittin: An allergen of honeybee venom

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The presence of serum IgE antibodies to melittin was tested by the radioallergosorbent test (RAST). Melittin, the principal protein of honeybee venom, was isolated by gel filtration on Sephadex G-75 and covalently bound to cyanogen bromide-activated microcrystalline cellulose. The melittin preparation was homogenous by immunoelectrophoresis with the use of rabbit antiserum to whole honeybee venom and by polyacrylamide electrophoresis in gels containing 1% sodium dodecyl sulfate. Elevated serum IgE antibodies to melittin (three times greater than binding by normal sera) were found in 7 of 24 honeybee venom-sensitive persons and in 5 of 20 nonsensitive beekeepers. In one venom-sensitive patient a particularly high titer of IgE antibody was found. The reaction between solid-phase melittin and IgE antibody could be inhibited by fluid-phase melittin but not by phospholipase A (PLA). Similarly, the reaction of IgE antibody with solid-phase PLA was inhibited by PLA but not by melittin. In passive transfer skin tests with the sensitive patient's serum, positive wheal-and-flare reactions were obtained in 3 nonallergic recipients following melittin challenge; appropriate controls were negative. These results indicate that melittin is an allergen in some honeybee venom-sensitive patients and in an occasional patient melittin may be a major allergen.

Hyaluronidase, phospholipase A, melittin, and apamin are all well recognized and well characterized protein constituents of honeybee venom (HBV).¹⁻⁴ Recent investigations of honeybee venom sensitivity, however, have focused attention on two components of honeybee venom, phospholipase A (PLA) and hyaluronidase, as major allergens.⁵⁻⁷ The major protein constituent of honeybee venom, melittin, is a basic 26 amino acid protein⁸ with strong surface-active and hemolytic properties.⁹ The allergenic activity of melittin has been investigated since 1956,¹⁰ and recent reports have indicated a possible role for melittin as a weak allergen.^{5-7, 11} Mackler and associates¹¹ observed painful late skin reactions when melittin was injected into honeybee-sensitive patients but they were unable to detect IgE antibodies in these patients' sera by the radioallergosorbent test (RAST). More recently, several investigators⁵⁻⁷ have reported that melittin is a weak or poor allergen. These prior

investigations, however, have not elucidated the frequency of occurrence or the magnitude of IgE antibodies in honeybee venom-sensitive persons or in nonsensitive, frequently stung, beekeepers. Our results indicate 29% of honeybee-sensitive subjects and 25% of nonsensitive beekeepers have elevated IgE antibodies to melittin, and in occasional honeybee-sensitive persons high titers of IgE antibody to melittin may be found.

MATERIALS AND METHODS

Sera

Sera were obtained from 20 nonallergic Minnesota beekeepers and 24 honeybee venom-sensitive members of beekeepers' families.¹² HBV-sensitive subjects exhibited an intense local reaction plus any one of the following systemic reactions: urticaria, angioedema, wheezing, rhinitis, or vascular collapse. Sera were also obtained from 9 patients with a history of ragweed hay fever.

Antisera

Rabbit anti-whole bee venom was prepared by subcutaneous injection of 2.8 and 5 mg of HBV (Champlain Valley Apiaries, Middlebury, Vt.) in complete Freund's adjuvant 1 mo apart. Rabbits were injected subcutaneously 1 mo later with 2.8 mg of HBV in incomplete Freund's adjuvant and 9 mo later with a series of intravenous injections containing a total of 7.8 mg alum-precipitated HBV. Sera were harvested 24 days after the final dose of HBV.

Fractionation of honeybee venom

Honeybee venom, grade 1 (Sigma Chemical Co., St. Louis, Mo.), was fractionated by gel filtration through a

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calibrated 2.5×200 cm column of Sephadex G-75 (Pharmacia, Piscataway, N. J.) equilibrated with 0.1 M ammonium formate (pH 4.5). Eight separate peaks were identified as described by Shepherd and associates.⁴ Elution values for PLA corresponded to a molecular weight of 14,000 to 16,000, while those for melittin corresponded to a molecular weight of 1,000 to 4,000. Fractions from the Sephadex G-75 column fractionation of HBV were analyzed for enzymatic activity on agarose egg blood plates.¹³ Briefly washed erythrocytes and an egg yolk suspension were added to agarose and poured into Petri dishes. After the agarose had solidified, wells 3 mm in diameter were punched and filled with 20 μ l of column fractions. PLA-containing fractions produced lysis of erythrocytes and clearing of egg yolk suspension, whereas melittin fractions produced erythrocyte lysis without clearing of egg suspension.

Melittin and PLA-rich fractions (as determined by the criteria above) were lyophilized and the purity was assessed by the following methods:

1. Analyses by electrofocusing on pH 3 to 10 thin-layer 5% polyacrylamide gels¹⁴ revealed two bands for PLA fractions at pIs of 8.78 and 9.15, respectively. Comparison of our PLA preparation with commercial PLA (Sigma Chemical Co., St. Louis, Mo.) showed that both gave virtually identical banding patterns. Analysis of melittin revealed a single heavily staining band at the cathodal end of the gel; an identical pattern was seen with commercial melittin (Sigma Chemical Co., St. Louis, Mo.). The pI for melittin was 10.0 or greater.

2. Immunoelectrophoretic analysis of the PLA and melittin showed single bands with rabbit anti-whole bee venom. In addition, melittin preparations also showed the non-immune precipitin arc described by Shepherd and co-workers.⁴

3. Analysis of PLA by electrophoresis in 8% polyacrylamide gels containing 1% sodium dodecyl sulfate¹⁵ showed two distinct bands corresponding to 14,000 to 19,000 molecular weight. Melittin fractions revealed a single band with a molecular weight less than 12,500.

Measurement of IgE antibodies

IgE antibodies were measured by the RAST.¹⁶ Solid-phase antigens were prepared by covalently linking melittin, PLA, and fractions from the Sephadex G-75 separation of HBV to either filter paper discs or microcrystalline cellulose. Filter paper discs were utilized solely to determine IgE antibody to chromatography fractions as the small quantities of protein per fraction were more conveniently bound to the filter paper discs. Samples of fractions from the G-75 Sephadex column and melittin, prepared by gel filtration of HBV, were coupled to 6.3-mm filter paper discs (Schleicher and Schuell, Keene, N. H.) as previously described.¹⁷ Melittin and PLA purified by gel filtration as noted above were coupled to microcrystalline cellulose (Brinkmann, Westbury, N. J.) as described earlier^{18, 19} with the following modifications: (1) 50 mg of melittin or PLA was coupled to 500 mg microcrystalline cellulose, and (2) 500 μ l of column fractions (and 1:10, 1:100, and 1:1,000 dilutions, thereof, in 0.1 M ammonium formate pH 4.5) were coupled to 4 activated filter paper discs. Direct RAST

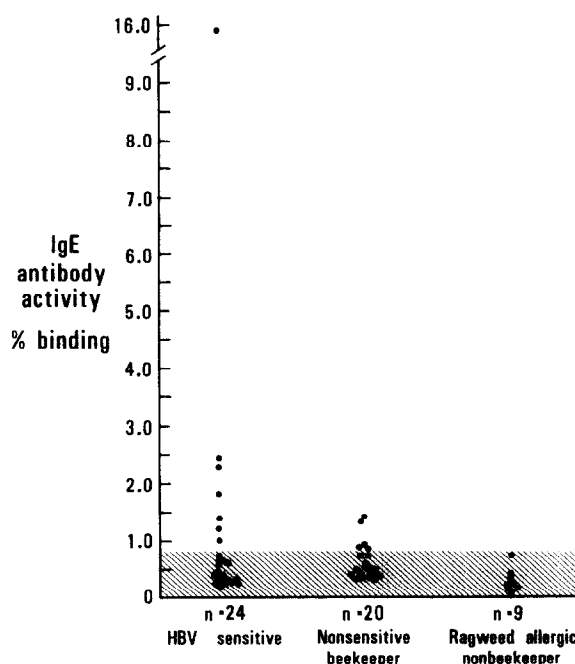


FIG. 1. IgE antibody activity to melittin in the sera of 20 nonsensitive Minnesota beekeepers, 24 honeybee venom-sensitive patients, and 9 ragweed-allergic nonbeekeepers. Results are expressed as the ratio of ¹³¹I counts bound to the melittin cellulose polymer divided by the total number of counts. Sera within 300% of the mean of ragweed-allergic control patients are considered negative (shaded area). Sera >300% of control (7 HBV-sensitive subjects and 5 nonsensitive beekeepers) showed significant levels of IgE antibody to melittin. One HBV-sensitive patient (406) demonstrated extremely high levels of IgE antibody to melittin. The nonsensitive beekeepers' IgE levels varied from 12 to 2,480 ng/ml with a mean of 700 ng/ml. The sensitive beekeepers' IgE levels varied from 52 to 7,540 ng/ml, a mean of 837 ng/ml. The ragweed-allergic nonbeekeepers' IgE levels varied from 862 to 6,500 ng/ml with a mean of 1,865 ng/ml. Despite the higher IgE levels in the ragweed-allergic control group, little binding to the melittin cellulose polymer was seen, indicating the specificity of the melittin cellulose polymer for IgE antibody to melittin.

determinations, as well as RAST inhibitions, were carried out as previously described.^{17, 18} Results were expressed as percent of total radioactive counts bound to the polymer.

Skin tests

Skin tests with melittin were performed in 6 nonallergic nonbeekeeper subjects to determine its irritant potential. Concentrations of melittin spanning a range from 100 μ g/ml to 1 ng/ml in sterile isotonic saline (Travenol Laboratories, Deerfield, Ill.) were injected intradermally (0.02 ml), and the resulting reactions at 15 min were measured and compared to the saline control. Passive transfer skin test experiments were conducted in 3 normal subjects with the use of serum from a melittin-sensitive patient (406). This serum was sterile and did not contain hepatitis B antigen as measured by a radioimmunoassay procedure. Serum, 1/10 ml, was injected intradermally into each of 6 sites in the 3

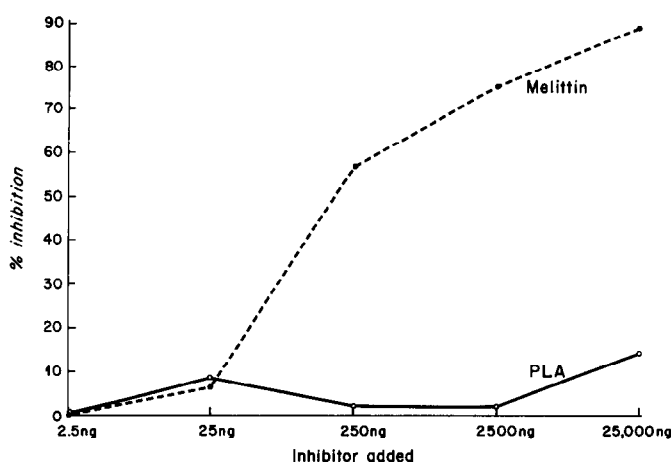


FIG. 2. Inhibition by fluid-phase PLA and melittin of the binding of IgE antibody contained in 406's serum to a melittin cellulose polymer. Percent inhibition is expressed on the ordinate. Amounts of inhibitor (fluid-phase PLA or melittin) are expressed on the abscissa. Fifty percent inhibition of the binding of 406's serum to melittin cellulose occurs at addition of 240 ng of fluid-phase melittin, whereas at 25,000 ng of fluid-phase PLA added less than 15% inhibition is noted.

recipients. Sensitized and control sites were challenged after 48 hr with 0.02 ml of melittin, 1, 0.1, and 0.01 $\mu\text{g/ml}$, and wheal-and-flare reactions were measured at 15 min.

RESULTS

The levels of IgE antibodies to melittin in 53 subjects are shown in Fig. 1. Elevated IgE antibodies (greater than 3 times the binding of ragweed-allergic nonbeekeepers' control sera) were found in 7 of 24 (29%) honeybee venom-sensitive patients and in 5 of 20 (25%) nonsensitive beekeepers. In one honeybee-sensitive individual (406) IgE antibody to melittin was markedly elevated.

To exclude the possibility that the melittin contained trace amounts of PLA and the results were due to this contamination, RAST inhibition titrations were carried out with the high-binding serum (406). In the first stage of the RAST, 406's serum and increasing amounts of inhibitor, PLA or melittin, were combined prior to the addition of the cellulose melittin polymer, as shown in Fig. 2. Marked inhibition of the binding of the IgE antibody to the melittin cellulose polymer was seen with increasing concentrations of fluid-phase melittin, and 50% inhibition occurred with 240 ng added melittin. In contrast, only minimal inhibition (less than 15%) occurred when 25,000 ng of PLA was added. To test further the possibility that the melittin preparation was contaminated with PLA, the reaction between IgE antibody to PLA and cellulose PLA was tested with the use of varying concentrations of melittin and PLA as inhibitors. In this experiment, 406's serum was used as the source of IgE antibody to PLA. As is shown in Fig. 3, inhibition of binding of IgE antibody to PLA cellulose occurred and increased with larger amounts of fluid-phase PLA, and 50% inhibition occurred at 22,000 ng. In

contrast, 25,000 ng of melittin produced minimal (less than 1%) inhibition. The above evidence strongly supports the view that IgE antibody in the high-binding serum (406) was directed to melittin.

Further evidence that the IgE antibody in 406's serum was directed to melittin was shown by the pattern of reactivity of the serum with HBV fractionated on a Sephadex G-75 column, as shown in Fig. 4. With 406's serum, peaks of IgE antibody activity coincided with the peaks of PLA and melittin activity, while little IgE antibody activity to melittin was found in the serum of another HBV-sensitive individual.

In vivo evidence that melittin functions as an allergen was provided by passive transfer skin tests with serum from Patient 406. Because melittin releases histamine nonspecifically,⁷ skin test titrations were performed in 6 nonallergic volunteers to ascertain the irritant concentrations of the test solutions. Skin test reactions with melittin in all 6 volunteers were similar to those with the normal saline control at melittin concentrations up to 1.0 $\mu\text{g/ml}$. Results of passive transfer skin tests are shown in Table I. Wheal and erythema were noted in passive transfer sites (0.1 and 1 $\mu\text{g/ml}$ at 15 min after challenge) and were significantly greater than those at the control sites.

DISCUSSION

The role of melittin as an allergen of HBV has been investigated only recently. Hoffman and Shipman⁵ analyzed the RAST activity of gel filtration fractions of honeybee venom with serum from 15 HBV-allergic patients. Eight patients' sera reacted with the melittin fraction and had "some allergenic activity," but numerical data were not provided. These investigations concluded that PLA appeared to be the major

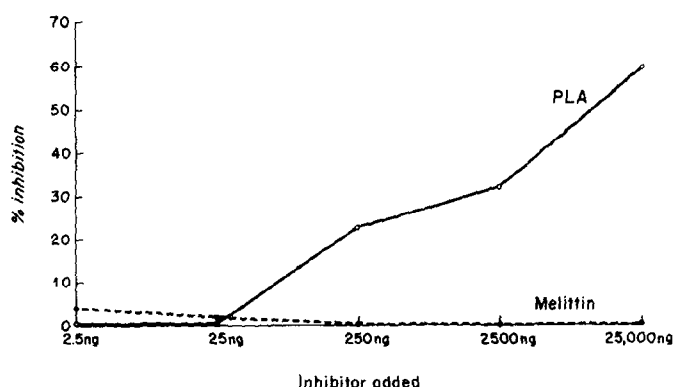


FIG. 3. Inhibition by fluid-phase PLA and melittin of the binding of IgE antibody contained in 406's serum to a PLA-cellulose polymer. Axes are labeled as in Fig. 2. Fifty percent inhibition of the binding of 406's serum to PLA cellulose occurs at 22,000 ng of fluid-phase PLA added, whereas at 25,000 ng of fluid-phase melittin less than 1% inhibition is noted.

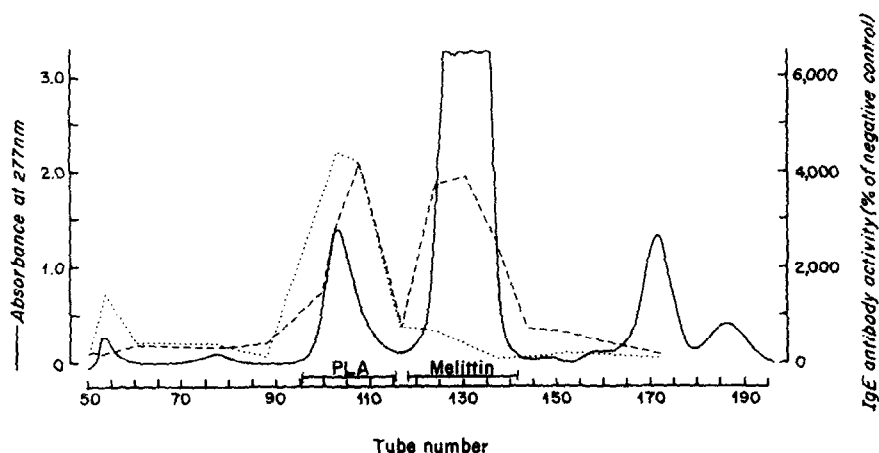


FIG. 4. IgE antibody activity of sera from 2 HBV-sensitive patients directed toward HBV components. The absorbance of the honeybee venom fractionated on Sephadex G-75 is shown by the solid line, and the pools corresponding to PLA and melittin are indicated. The fraction tube numbers are marked on the abscissa. IgE antibody activity as measured by RAST in 2 venom-sensitive subjects is illustrated by broken lines. Activity is measured as percent of a negative control serum (ordinate), e.g., if normal serum yielded 1.5% of counts bound and the patient's serum yielded 15% binding, then the IgE would be $15\% / 1.5\% \times 100 = 1,000\%$ of control. One HBV-sensitive patient, 406 (---) demonstrates peaks of IgE antibody activity which roughly correspond with peaks of PLA and melittin activity of the chromatogram, whereas another HBV-sensitive patient (...) demonstrates IgE antibody activity to PLA but little if any activity to melittin.

allergen of HBV whereas "melittin appeared to be a poor allergen." Sobotka and colleagues⁶ examined leukocyte histamine release in the leukocytes from 6 HBV-sensitive patients and found that all leukocytes were most sensitive to PLA. In addition, all leukocytes demonstrated significant histamine release with nanogram concentrations of melittin. Nonspecific histamine release by melittin was not seen with concentrations of 10 $\mu\text{g}/\text{ml}$; however, a 1% contamination of their melittin preparation with PLA could not be discounted as the cause for histamine release. Most recently, King and associates⁷ analyzed the allergenic activity of melittin obtained by gel filtration of HBV. Leukocytes from 6 HBV-allergic patients were shown to release histamine in response to melittin. Nonspecific histamine release was not seen with con-

centrations below 10 $\mu\text{g}/\text{ml}$, and histamine release could not be accounted for by the 0.01% PLA contamination of the melittin pool. The relative allergenic activity of melittin averaged one tenth that of whole HBV, and King and co-workers concluded that melittin possessed weak allergenic activity.

In our studies, elevated IgE antibody levels to melittin were found in the sera of 7 of 24 (29%) HBV-sensitive individuals and in 5 of 20 (25%) non-sensitive honeybee keepers. In 2 subjects high titers of IgE antibody to melittin were noted (a second person with high titers of IgE antibody to melittin has been identified, but the data are not presented here). PLA contamination of the melittin polymer which could have accounted for the binding of IgE antibody was not detected by electrofocusing, immunoelec-

TABLE I. Passive transfer skin tests in 3 nonallergic volunteers elicited 48 hr after injection of serum from Patient 406 and a nonallergic control serum

Subject	Patient 406 serum			Control serum		
	0.01 $\mu\text{g/ml}$ melittin	0.1 $\mu\text{g/ml}$ melittin	1.0 $\mu\text{g/ml}$ melittin	0.01 $\mu\text{g/ml}$ melittin	0.1 $\mu\text{g/ml}$ melittin	1.0 $\mu\text{g/ml}$ melittin
No. 1						
Wheal (mm)	3 $\times$ 3	4 $\times$ 3	4 $\times$ 5	0	0	4 $\times$ 3
Flare (mm)	0	7 $\times$ 6	12 $\times$ 11	0	0	0
No. 2						
Wheal (mm)	2 $\times$ 3	3 $\times$ 3	5 $\times$ 5	0	0	0
Flare (mm)	0	4 $\times$ 4	6 $\times$ 6	0	0	0
No. 3						
Wheal (mm)	2 $\times$ 3	3 $\times$ 2	4 $\times$ 3	0	0	0
Flare (mm)	0	15 $\times$ 5	25 $\times$ 25	0	0	0

Three concentrations of melittin were used to challenge Patient 406 and control serum sites.

trophoresis, polyacrylamide gel electrophoresis, or in two RAST inhibition experiments. As additional evidence for the allergenicity of melittin, positive passive transfer skin tests were elicited in 3 nonallergic volunteers with the use of serum from a patient with a high titer of IgE antibody to melittin. Unlike those in previous studies, our findings suggest melittin is an important allergen in some HBV-sensitive subjects and, in addition, demonstrate the prevalence of IgE antibodies in HBV-allergic and beekeeper populations. Whereas PLA and hyaluronidase are undoubtedly major allergenic components of HBV, melittin and other allergenic components previously unrecognized²⁰ may be of great importance in certain HBV-allergic individuals. These findings have bearing on both the question of the best reagent for detection of IgE antibodies to HBV and the choice of the proper reagent for immunotherapy.

Authors' note added in proof. Since this paper was submitted for publication, Dr. Anne K. Sobotka, of the Clinical Immunology Division, The Good Samaritan Hospital, Johns Hopkins University, has examined the sera from both patients with high titers of IgE antibody to melittin described in this report. Melittin was capable of releasing histamine following passive leukocyte sensitization with each serum at melittin concentrations of $<1.0 \mu\text{g/ml}$.

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