

Allergenic potency of bee antigens measured by RAST inhibition

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Summary

The potency of various bee antigens including bee venom, several whole bee body extracts and fractions of bee venom was studied using the RAST inhibition method. As compared to whole bee body extract, bee venom was a much more potent inhibitor of both bee venom and whole body RAST, suggesting that venom has a greater capacity to bind specific bee IgE antibodies. Whole body extracts also varied substantially in their inhibiting activity. Phospholipase A and hyaluronidase were the most potent of the bee venom fractions suggesting their potential use as an assay for standardization of insect extracts.

Introduction

For many years extracts prepared from whole insect bodies have been used for the diagnosis and treatment of individuals with stinging insect allergy. The basis for the use of this type of extract was the observation by Benson (1939) that the antigen responsible for allergic reactions was present throughout the body of the insect as well as in the insect venom. This concept was challenged in subsequent studies. Shulman *et al.* (1966) described the presence of a 'tissue specific' antigen in bee venom and not in the remainder of the whole body. More recent reports have suggested that bee venom is a much more specific allergen than whole bee body extract for the diagnosis and treatment of bee sensitive individuals (Busse *et al.*, 1975; Hunt *et al.*, 1975; Lichtenstein, Valentine & Sobotka, 1974; Light *et al.*, 1976). Furthermore, bee venom has been fractionated into a number of relatively pure components (Shepherd, Elliott & Arbesman, 1974) and it has been suggested that phospholipase A and perhaps hyaluronidase are major antigenic fractions (Light *et al.*, 1976; Sobotka *et al.*, 1974).

This report deals with the study of the activity of various bee antigens including bee venom, whole bee body extract, and bee venom fractions by the radioallergosorbent (RAST) inhibition technique. RAST inhibition has been proposed as an accurate assay of allergenic potency (Gleich *et al.*, 1974; Yman, Ponterius & Brandt, 1975).

Our results confirm the superiority of bee venom as diagnostic allergen and also show much variability in the potency of different whole bee body extracts.

Materials and methods

Bee antigens

Bee venom was obtained by electrical stimulation of bees following the method described by Benton, Morse & Stewart (1963).

Bee whole body extract (ARL) was prepared in our laboratory. Fifteen grams of whole bee bodies were added to 100 ml of buffered saline in a Waring Blender and mixed for 3 min in the cold room. The supernatant was removed by centrifugation at 16,000 rev/min for 5 min. This was clarified by further centrifugation in an ultracentrifuge at 35,000 rev/min for 1 hr. The total protein of this extract was determined by the Lowry method (Lowry *et al.*, 1951) and the PNU was assayed by the macro-Kjeldahl technique. Three other whole bee body extracts were obtained from pharmaceutical companies. The weight to volume and PNU values for these extracts were supplied by the manufacturer. The total protein was measured by Biuret (Gornall, Bardawill & David, 1949), adjusted for the effect of pigments (Rapp, Shulman & Arbesman, 1962).

The following fractions of bee venom were obtained in sufficient quantity and purity for study: phospholipase A, apamine, mellitin and hyaluronidase (Shepherd *et al.*, 1974).

Radioallergosorbent (RAST) Inhibition

Sera containing relatively high levels of specific IgE to both bee venom and phospholipase A as measured by direct RAST were collected from six bee sensitive patients. These sera were pooled and served as our positive standard reference serum.

The 50% RAST inhibition was determined by the following method. Cellulose paper discs were coupled by cyanogen bromide to bee venom, whole bee body extract (ARL) or phospholipase A. The optimal concentration of each antigen for coupling was determined in preliminary direct RAST experiments using the reference pool. Various concentrations of each of the above antigens were coupled to different sets of paper cellulose discs. The least concentration that produced the highest RAST level was used. The final amounts (in a volume of 20 ml of buffer) used in these studies for coupling 200 discs for each antigen were 20 mg of bee venom, 2 ml of the 15% whole bee body extract or 5 mg of phospholipase A.

Serial ten-fold dilutions of each of the inhibiting extracts in 2% HSA were prepared and 150 μ l placed in duplicate rows of twelve (polystyrene) test tubes. The last tube in each row contained only the diluent (2% HSA). Fifty microlitres of the reference serum pool were then added to each tube. The mixture was shaken vigorously and kept at 4°C overnight. Either the bee venom, whole bee body extract or phospholipase A coupled disc was then placed in each tube and kept at room temperature for 3 hr. After several washings with the buffered solution and removal of the unbound antigen antibody mixture by suction, 50 μ l of goat radiolabelled anti-IgE were added to each tube. The tubes were capped and kept overnight at 4°C. Following several washings to remove unbound radioactive anti-IgE, the radioactivity on each disc was measured in a gamma counter.

The amount of each bee antigen required to produce 50% RAST inhibition was

determined in terms of weight/volume, PNU and total protein. A logarithmic plot was used.

Results

Measurements of whole bee body extracts

The total protein, PNU, and weight/volume of each whole bee body extract are listed in Table 1. There was no relationship between these parameters in any of the four extracts. Extract nos 1 and 4 were 15% extracts but the PNU/ml were 70,000 and 40,000, and the total protein 3,350 and 2,200 $\mu\text{g/ml}$, respectively. There were similar differences in the two 10% extracts, no. 2 and no. 3.

Table 1. Content of whole bee body extracts

Extract	Weight volume (%)	Protein nitrogen (units/ml)	Total protein ($\mu\text{g/ml}$)
1*	15	70,000	3,350
2	10	25,000	1,250
3†	10	18,000	2,300
4†	15	40,000	2,200

* ARL: prepared in our laboratory and used to couple discs.

† From the same commercial source; different lot numbers and prepared at different times.

Table 2. Amount of whole bee body extracts required for 50% inhibition of whole bee body RAST

Extract	Weight/volume	Protein nitrogen (units/ml)	Total protein ($\mu\text{g/ml}$)
1	1–11,500	42	1.9
2	1–310	780	42
3	1–18	10,000	1,350
4	1–13.5	19,000	1,050

RAST inhibition by whole bee body (WBB) extracts

(a) *WBB coupled discs.* The three commercial extracts and the homologous WBB antigen (ARL) on the disc were used as inhibiting antigens. In the average inhibition experiment there was a range of 3,000 ct/min from 0 to 90% inhibition. Duplicate samples agreed within 15%.

The results of 50% RAST inhibition by each of the four extracts are summarized in Table 2 and an example of the inhibition curve shown in Fig. 1. There was a wide range of amount of each extract required to produce 50% inhibition as measured by w/v (1–11,500 to 1–13.5), PNU/ml (42–19,000) and protein $\mu\text{g/ml}$ (2.0–1,350). The homologous antigen (ARL) appeared to be the most potent as measured by each parameter; extract no. 2 was the next most active. Considerably more of extract nos

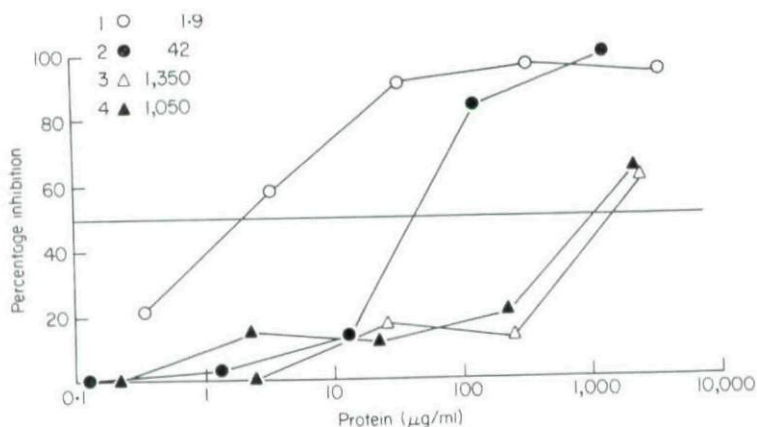


Fig. 1. Inhibition of bee body RAST by bee body extracts. ○—○, No. 1 whole bee body extract (15%) ARL; ●—●, no. 2 commercial whole bee body extract (10%); □—□, no. 3 commercial whole bee body extract (10%); ▲—▲, no. 4 commercial whole bee body extract (15%). Ordinate, percentage of inhibition; abscissa, total protein μg/ml. Amount of each antigen that produces 50% inhibition is shown on left upper corner.

3 and 4, purchased from the same commercial source but prepared at different times, were required to produce 50% inhibition.

(b) *Bee venom coupled discs.* The same inhibiting whole bee body extracts and standard reference serum were used to study inhibition of bee venom coupled discs. The data obtained are illustrated in Fig. 2 and summarized in Table 3. The results were quite similar to that found employing whole bee body coupled discs. In these experiments, extract no. 2 was essentially equivalent to extract no. 1 (ARL). Extract nos 3 and 4 were similar to each other but much greater amounts were needed to produce 50% inhibition. For example, using total protein as the measuring parameter,

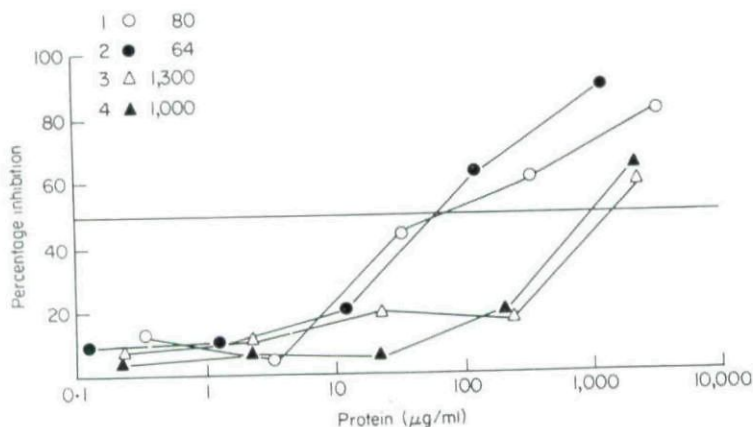


Fig. 2. Inhibition of bee venom RAST by bee body extract. ○—○, No. 1 whole bee body extract (15%) ARL; ●—●, no. 2 commercial whole bee body extract (10%); □—□, no. 3 commercial whole bee body extract (10%); ▲—▲, no. 4 commercial whole bee body extract (15%). Ordinate, percentage of inhibition; abscissa, total protein μg/ml. Amount of each antigen that produces 50% inhibition is shown on left upper corner.

Table 3. Amount of whole bee body extracts required for 50% inhibition of bee venom RAST

Extract	Weight/volume	Protein nitrogen (units/ml)	Total protein ($\mu\text{g/ml}$)
1	1-270	1,700	80
2	1-210	1,300	64
3	1-18	9,100	1,300
4	1-14	19,000	1,000

80 and 64 $\mu\text{g/ml}$ of extract nos 1 and 2, and 1,300 and 1,000 $\mu\text{g/ml}$ of extract nos 3 and 4 caused 50% inhibition.

(c) *Phospholipase A coupled discs.* The results of 50% inhibition of PLA coupled discs using the same whole body extracts and standard reference serum pool are illustrated in Fig. 3 and summarized in Table 4. Again, there was a broad range in the amount of whole bee body extracts required to produce 50% inhibition. Extract no. 1

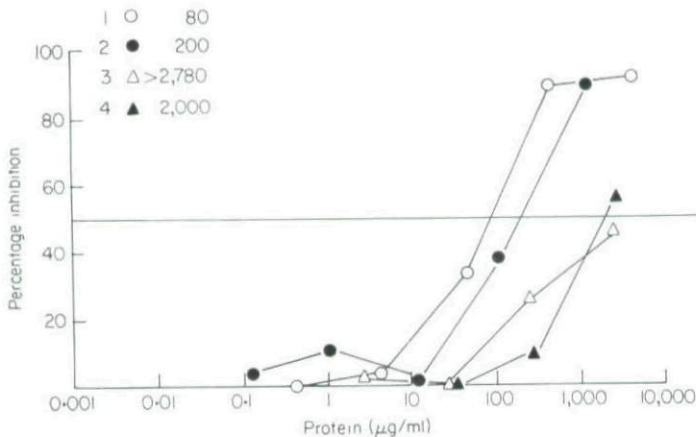


Fig. 3. Inhibition of phospholipase A RAST by bee body extract. ○—○, No. 1 whole bee body extract (15%) ARL; ●—●, no. 2 commercial whole bee body extract (10%); □—□, no. 3 commercial whole bee body extract (10%); ▲—▲, no. 4 commercial whole bee body extract (15%). Ordinate, percentage of inhibition; abscissa, total protein $\mu\text{g/ml}$. Amount of each antigen that produces 50% inhibition is shown on left upper corner.

Table 4. Amount of whole bee body extract required for 50% inhibition of phospholipase A RAST

Extract	Weight/volume	Protein nitrogen (units/ml)	Total protein ($\mu\text{g/ml}$)
1	1-350	1,000	80
2	1-56	4,200	200
3	1-10	N.D.*	> 2,780
4	1-6.7	40,000	2,000

* Not done

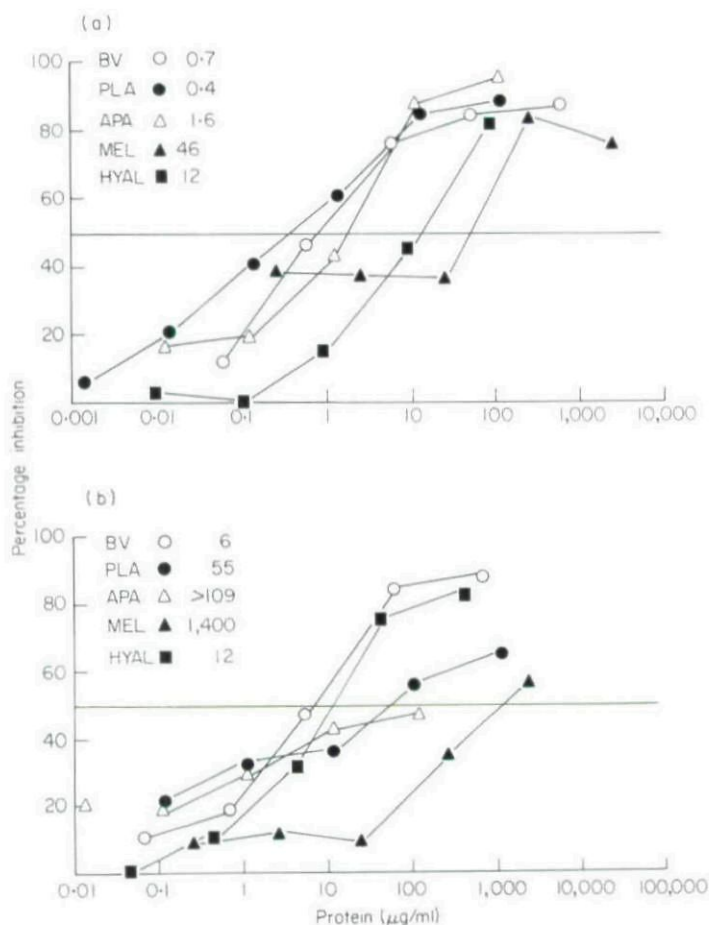


Fig. 4. Inhibition of bee body and bee venom RAST by bee venom and its fractions. ○—○, Bee venom (BV); ●—●, phospholipase A (PLA); □—□, apamine (APA); ▲—▲, mellitin (MEL); ■—■, hyaluronidase (HYAL). Ordinate, percentage of inhibition; abscissa, $\log_{10}$ protein $\mu\text{g/ml}$. (a) Bee body coupled discs; (b) bee venom coupled discs.

Amount of each antigen that produces 50% inhibition is shown on left upper corner.

(ARL) required the least amount to cause 50% inhibition. Extract no. 2 was the next most potent. The rank order was about the same when extract potency was measured by PNU/ml and total protein $\mu\text{g/ml}$.

RAST inhibition by bee venom and its fractions

(a) *Whole bee body coupled discs.* The results of 50% inhibition of whole bee body RAST by bee venom, phospholipase A, apamine, mellitin and hyaluronidase, expressed in $\mu\text{g/ml}$ of protein, are diagrammatically shown in Fig. 4a. The amount of each inhibiting antigen that was necessary to produce 50% inhibition was as follows: 0.4 $\mu\text{g/ml}$ of PLA, 0.7 $\mu\text{g/ml}$ of bee venom, 1.6 $\mu\text{g/ml}$ of apamine, 12 $\mu\text{g/ml}$ of hyaluronidase and 46 $\mu\text{g/ml}$ of mellitin. As noted previously, 1.9 $\mu\text{g/ml}$ of the homologous whole bee body extract (ARL) was necessary to cause 50% inhibition.

(b) *Bee venom coupled discs.* The results of 50% inhibition of bee venom coupled

discs are shown in Fig. 4b. The amount of each inhibiting antigen that was necessary to produce 50% inhibition was as follows: 6.0 µg/ml of bee venom, 12 µg/ml of hyaluronidase, 55 µg/ml of phospholipase, > 109 µg/ml of apamine and 1,400 µg/ml of mellitin.

Much greater amounts of whole bee body extract than bee venom were required for 50% inhibition of bee venom discs. Furthermore, there was considerable variation in the inhibiting potency of the different whole body extracts ranging from 64 to 1,300 µg/ml of protein.

Hyaluronidase was the most potent of the bee venom fractions. As compared to bee venom, twice as much hyaluronidase and almost ten times as much PLA was needed to cause 50% inhibition. Apamine and mellitin caused little inhibition.

Much greater amounts of fractions (PLA, apamine and mellitin) and bee venom were required to produce 50% inhibition of bee venom discs as compared to bee whole body discs. However, hyaluronidase was an exception. The same amount of this fraction caused 50% inhibition of both coupled discs.

(c) *Phospholipase A coupled discs.* Fifty per cent RAST inhibition of PLA discs was produced with 0.18 µg/ml of PLA, 1.7 µg/ml of bee venom and whole bee body extract ranging from 80 to over 2,700 µg/ml. Again it was the extract prepared in this laboratory which was the most potent of the whole body extracts.

Discussion

The RAST inhibition method as applied to the insect allergen system provided reliable results. Duplicate samples showed good agreement and these results were reproducible in repeated tests.

Direct skin tests with whole body extracts and bee venom and its fractions were not performed. The interpretation of the RAST inhibition results as a measurement of allergenic potency is based upon other studies (Arbesman, Wypych & Reisman, 1976; Gleich *et al.*, 1974).

The present studies show that whole bee body extracts are much less capable of binding the IgE antibody in the standard reference pool than bee venom. Greater amounts of body extracts than bee venom were needed for inhibition of both the bee body and bee venom RAST. Furthermore, these whole bee body extracts vary considerably in potency as measured by 50% RAST inhibition, without any relationship to presently used standards of measurements such as PNU or weight to volume. These differences in the whole body extracts might explain some of the conflicting results that have been reported in their use as diagnostic and therapeutic agents.

Phospholipase A and hyaluronidase gave significant inhibition of the bee venom RAST. Minimal inhibition was noted with apamine and mellitin. Phospholipase A has been suggested as a major allergen in bee venom (Sobotka *et al.*, 1974). Previous studies in our own laboratory indicate that other fractions may also be important (Light *et al.*, 1976). We found that bee venom with no detectable phospholipase A after immunoadsorption with anti PLA antiserum could still produce inhibition of the RAST with bee venom coupled discs (Arbesman, Wypych & Reisman, 1975). These present studies suggest that hyaluronidase may also be an important fraction. In order to check on specificity of the inhibition by hyaluronidase, two controls were performed. Hyaluronidase was used as an inhibiting antigen in the ragweed RAST system. There was no inhibition of the ragweed RAST even with amounts of hyaluronidase 100-fold greater than used in the present experiments. In addition, commercial hyaluronidase was used to determine whether its enzyme activity could produce inhibition of the

bee-anti-bee venom reactions. The commercial hyaluronidase is prepared from bull's testes and gave no inhibition.

The same amount of hyaluronidase caused 50% inhibition of both bee body and bee venom RAST. However, considerably less bee venom and its fractions, phospholipase A, apamine and mellitin, were needed to cause inhibition of bee body RAST as compared to bee venom RAST. This suggests that there is considerably less of these latter antigens in whole bee body extracts. Less phospholipase A than bee venom was required for 50% inhibition of whole bee body RAST. This suggests that some phospholipase A may be destroyed in the preparation of whole bee body extracts, perhaps in keeping with the decreased allergenic potency of these extracts.

The use of phospholipase A as a coupling antigen to cellulose discs for RAST inhibition had no advantages over the use of bee venom.

Our findings suggest that the use of bee venom coupled discs in the RAST inhibition technique might be a good method for standardization of extracts.

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