

# Immediate allergic reactions to *Myrmecia* ant stings: immunochemical analysis of *Myrmecia* venoms

M. D. STREET\*, G. R. DONOVAN\*, B. A. BALDO\*† and S. SUTHERLAND‡

\*Kolling Institute of Medical Research, Royal North Shore Hospital of Sydney, St Leonards, NSW,

†Department of Medicine, University of Sydney, Sydney, NSW, ‡Commonwealth Serum Laboratories, Parkville, Victoria, Australia

## Summary

IgE antibody reactivities to the venoms of *Myrmecia pilosula*, *Myrmecia nigrocincta*, *Myrmecia tarsata*, *Myrmecia pyriformis*, *Myrmecia simillima* and *Myrmecia gulosa* have been identified in sera from subjects allergic to ant venom. Sera with IgE reactivity to only a single *Myrmecia* venom most often recognize *M. pilosula* venom although all six venoms appear capable of inducing IgE antibodies. The six different *Myrmecia* venoms were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), Western blotted to nitrocellulose and probed with sera from ant venom-allergic subjects. Four to six IgE-binding components ranging in size from 2 to 25 kDa were identified in each of the venoms. Similarities in molecular weights of the IgE-binding components exist and close taxonomic relationships between the species suggest that common or similar peptides may be present in the different venoms.

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## Introduction

The sting from ants of the genus *Myrmecia* (Fig. 1), can cause a range of allergic responses including anaphylaxis in certain sensitized individuals [1–3].

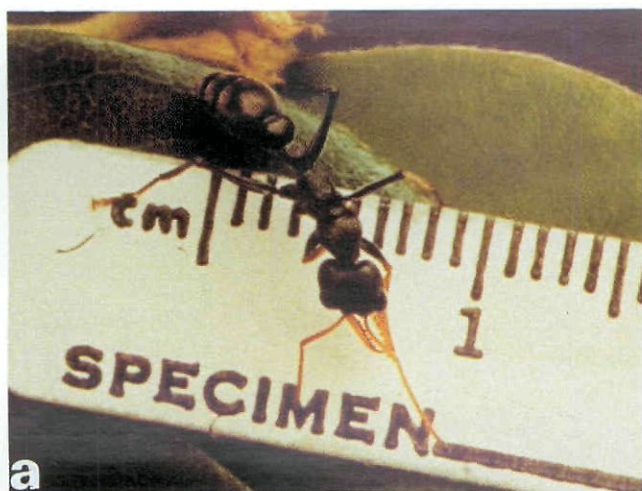
Ants of this genus, consisting of approximately 89 recognized species [4], are found only in Australia with one exception, New Caledonia [4,5]. The genus *Myrmecia* is part of the family Formicidae (Order Hymenoptera) (Fig. 2), which encompasses all of the world's ant species [5], including the 'imported fire ant' *Solenopsis invicta*.

Correspondence: Dr B. A. Baldo, Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW 2065 Australia.

Although the precise incidence of anaphylaxis is unknown, the incidence of severe allergic reaction to stings is increasing [2]. The Jumper Ant Register has collected data on 224 patients with a total of 454 sting episodes over a period of 5 years. A total of 239 episodes were described as life-threatening situations representing a serious reaction rate of 52.6%. (Dr J. Weiner, Asthma and Allergy Unit, Alfred Hospital, Melbourne, Victoria, Australia; personal communication).

The ants' aggressive behaviour and propensity to sting is a major problem for those individuals hypersensitive to *Myrmecia* venoms. The problem is comparable to the menace presented by the imported fire ant in southern USA. While extensive studies of allergy to *S. invicta*

**Figure 1.** Six different species of ants from the genus *Myrmecia*. (a) *Myrmecia pilosula*; (b) *Myrmecia nigrocincta*; (c) *Myrmecia tarsata*; (d) *Myrmecia pyriformis*; (e) *Myrmecia simillima*; (f) *Myrmecia gulosa*. The genus *Myrmecia* consisting of approximately 89 recognized species, is distributed throughout Australia [4]. Of the six species presently under investigation, *M. pilosula* (a) and *M. nigrocincta* (b) are the smallest ants being approximately 1–1.5 cm in length. These two species can be easily distinguished by *M. pilosula*'s all black body, including head and gaster (abdomen), and *M. nigrocincta*'s alternating black and orange-red coloured body segments. The four other species are all approximately 2–2.5 cm in length, with *M. gulosa* (f) being recognizable by its orange-red body colouring and black-tipped gaster. *M. tarsata* (c) with its all black body and yellow-tipped gaster is distinguishable from *M. pyriformis* (d) and *M. simillima* (e). *M. pyriformis*' dark brown body colouring, including mandibles and antennae, distinguishes it from *M. simillima* which has an all black body and light orange mandibles and antennae. Photographs by N. Fisher, Medical Illustrations, Royal North Shore Hospital, NSW, Australia.



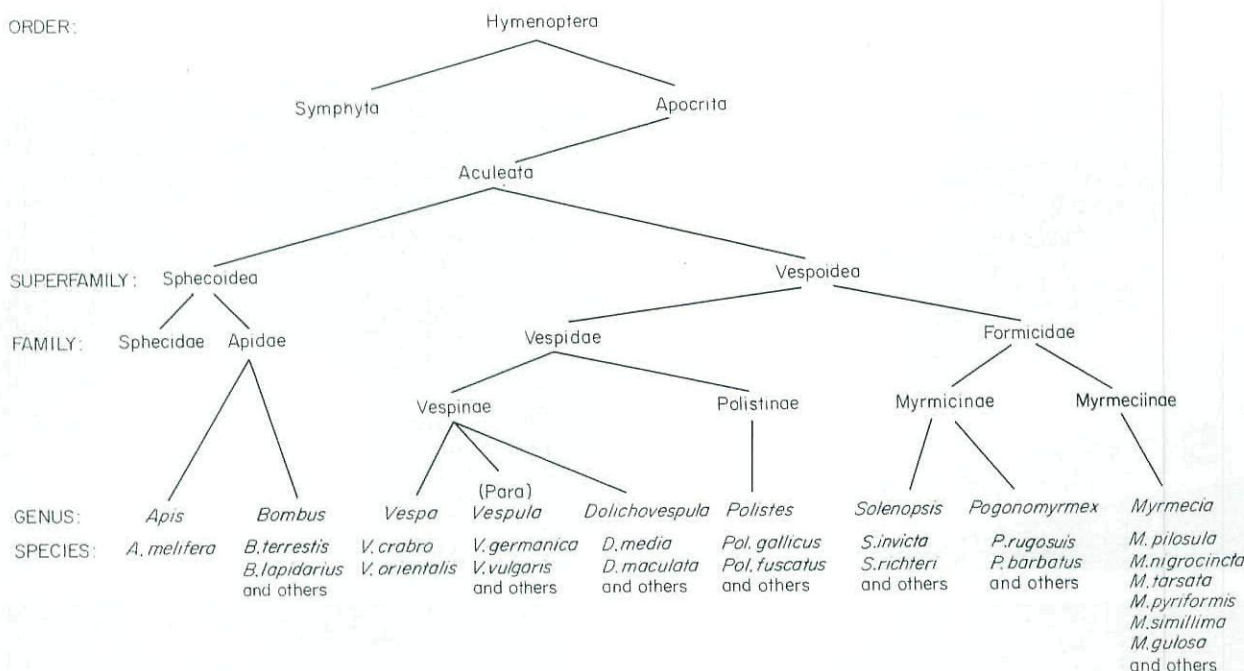


**Figure 2.** Scheme depicting the phylogenetic relationships between the six *Myrmecia* species examined and other species of the order Hymenoptera [4, 21 and T. E. Bellas, I. D. Naumann, R. W. Taylor, Personal Communications, Division of Entomology, CSIRO, Canberra, Australia).

venom have been performed, including detailed examination and characterization of the venom allergens [6–14], only limited information is available on the venoms of *Myrmecia* ants.

Recent investigations of allergic reactions to *Myrmecia pilosula* identified five IgE-binding components in the venom of this ant [2, 15]. Cloning of a major allergen, *Myr p I*, was also achieved [15].

In this report, results of studies designed to investigate allergic reactions to the venoms of six different *Myrmecia* species, and to characterize the venoms immunologically, are presented.

## Materials and methods

### Ants and ant venom extracts

*M. pilosula* and *M. pyriformis* were collected at Wentworth Falls, NSW, *M. nigrocincta* and *M. tarsata* from the Strickland State Forest, Gosford, NSW and *M. simillima* and *M. gulosa* from the Eloura Bushland, Sydney, NSW. Venom samples from the six *Myrmecia* species were prepared as described previously [2].

Freeze-dried venom samples were dissolved in de-ionized water at a concentration of 2 mg ml<sup>-1</sup>. Protein concentrations of aqueous venom samples were measured using the BCA Protein Assay (Pierce, Rockford, IL, USA).

### Detection of venom-reactive IgE antibodies (RAST)

Sera from subjects allergic to ant venom(s) were tested for the presence of IgE antibodies to the six different *Myrmecia* venoms. Aliquots of sera (50 µl) were incubated with 6 mm nitrocellulose discs (0.45 µm pore size, Schleicher and Schull, Dassel, Germany) coated with 2.5 µg of venom proteins per disc [2, 15, 16]. Bound IgE antibodies were detected using <sup>125</sup>I-anti-human IgE (Bioclone, Australia), c 30 000 cpm/tube, and the results expressed as the percentage uptake of the radioactive label. An IgE 'free' control (human cord serum) and an IgE negative control (a serum from an allergic subject, but with no *Myrmecia* venom-reactive IgE antibodies), were routinely included in these assays.

### Quantitative inhibition studies

Sera from *Myrmecia* venom-allergic subjects which reacted with more than one of the six different venoms examined, were tested for the presence of crossreactive IgE antibodies using an inhibition assay. Serum (25 µl), was incubated for 3 h at room temperature with 25 µl (5 µg/ml protein), of either *M. pilosula*, *M. nigrocincta*, *M. tarsata*, *M. pyriformis*, *M. simillima* or *M. gulosa* venoms. An *M. pilosula* venom disc, was added to each inhibition reaction and the mixture incubated a further 3 h at room

**Table 1.** IgE antibody reactivity in the sera of antivenom allergic subjects, to the venom of six different *Myrmecia* species

|                                                                | <i>Myrmecia pilosula</i> | <i>Myrmecia nigrocincta</i> | <i>Myrmecia tarsata</i> | <i>Myrmecia pyriformis</i> | <i>Myrmecia simillima</i> | <i>Myrmecia gulosa</i> | Two or more venoms† |
|----------------------------------------------------------------|--------------------------|-----------------------------|-------------------------|----------------------------|---------------------------|------------------------|---------------------|
| Number of sera with a positive*<br>IgE reactivity to the venom | 52                       | 2                           | 1                       | 4                          | 1                         | 0                      | 31                  |

\* An uptake of the radioactive label >2% was considered a positive result.

† Sera with positive IgE reactivities to two or more *Myrmecia* venoms.

temperature. After washing,  $^{125}\text{I}$ -anti-human IgE was added and the assay completed as above. Counts obtained from tubes containing serum but no added venom were used to calculate percentage inhibition by the venoms.

#### *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

Venom samples were prepared in a SDS-sample buffer containing 5% (v/v) 2-mercaptoethanol [15,17]. Samples were heated at 100°C for 10 min and cooled to room temperature prior to loading.

Proteins were separated on a 16.5% Total, 6% Cross-linker separating gel using a *N*-tris (hydroxymethyl)-methylglycine-SDS-polyacrylamide gel electrophoresis system [15,17].

Venom components to be electrophoretically transferred to nitrocellulose were separated on a 18 cm × 16 cm vertical gel (The Studier SE400, Hoefer Scientific Instruments, San Francisco, CA, USA). Venom solution (300 µl; 50 µg/ml protein) was loaded across the full width of the stacking gel. Polypeptide markers (Pharmacia, LKB Technology AB, Uppsala, Sweden), were run in tracks on either side of the sample well.

Venom samples for subsequent protein staining were separated using a vertical mini-gel system (Bio Rad Mini Protean II Dual Slab Cell, Richmond, CA, USA). Venom solution (20 µl; 500 µg/ml) was loaded into each well and molecular weight markers (Pharmacia, LKB Technology), covering the range 2–94 kDa, were run in separate wells.

#### *Electrotransfer to nitrocellulose and immunoprobings*

Separated venom components were electrophoretically transferred to nitrocellulose membrane (0.1 µm pore size, Schleicher and Schull), as previously described [15]. The electroblots were cut into 4 mm strips and probed with sera of *Myrmecia* ant venom-allergic subjects. IgE-

binding components were identified with  $^{125}\text{I}$ -anti-human IgE and autoradiography [15].

#### *Protein staining*

Polyacrylamide gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St Louis, MO, USA), or silver stained as described previously [18]. Gels and nitrocellulose transfers were also stained with 0.1% w/v amido black (Sigma), in 5% v/v acetic acid/29% (v/v) methanol or 0.1% (w/v) Ponceau S (Sigma), in 0.5% v/v acetic acid. Amido black stained gels and transfers were destained in 5% (v/v) acetic acid/29% (v/v) methanol and Ponceau S stained gels were destained in 0.5% acetic acid. Ponceau S stained filters were destained by rinsing twice in de-ionized H<sub>2</sub>O.

## Results

### *IgE antibody recognition of Myrmecia venoms by different allergic subjects*

Sera from subjects allergic to the stings of ants of the genus *Myrmecia* were examined to determine their IgE antibody reactivity to the venom preparations from six different *Myrmecia* species (Table 1). Results can be divided into two groups: sera that reacted with a single *Myrmecia* venom, not necessarily *M. pilosula* venom, and sera with IgE reactivities against two or more venoms.

In the former group, *M. pilosula* was the venom most often implicated. Recognition of the *M. pilosula* venom was also observed in 26 of the 31 sera from the second group who recognized two or more venoms (Fig. 3). However, sera in this group also recognized the other five venoms with a frequency similar to that of *M. pilosula* venom. Sera that reacted with only one venom, other than *M. pilosula* venom, occurred at low frequency (Table 1).

Direct binding studies with venoms of all six *Myrmecia* species using the 31 sera exhibiting multiple IgE reactivity are shown in Fig. 3. These data show that IgE antibody recognition by allergic subjects to the different ant

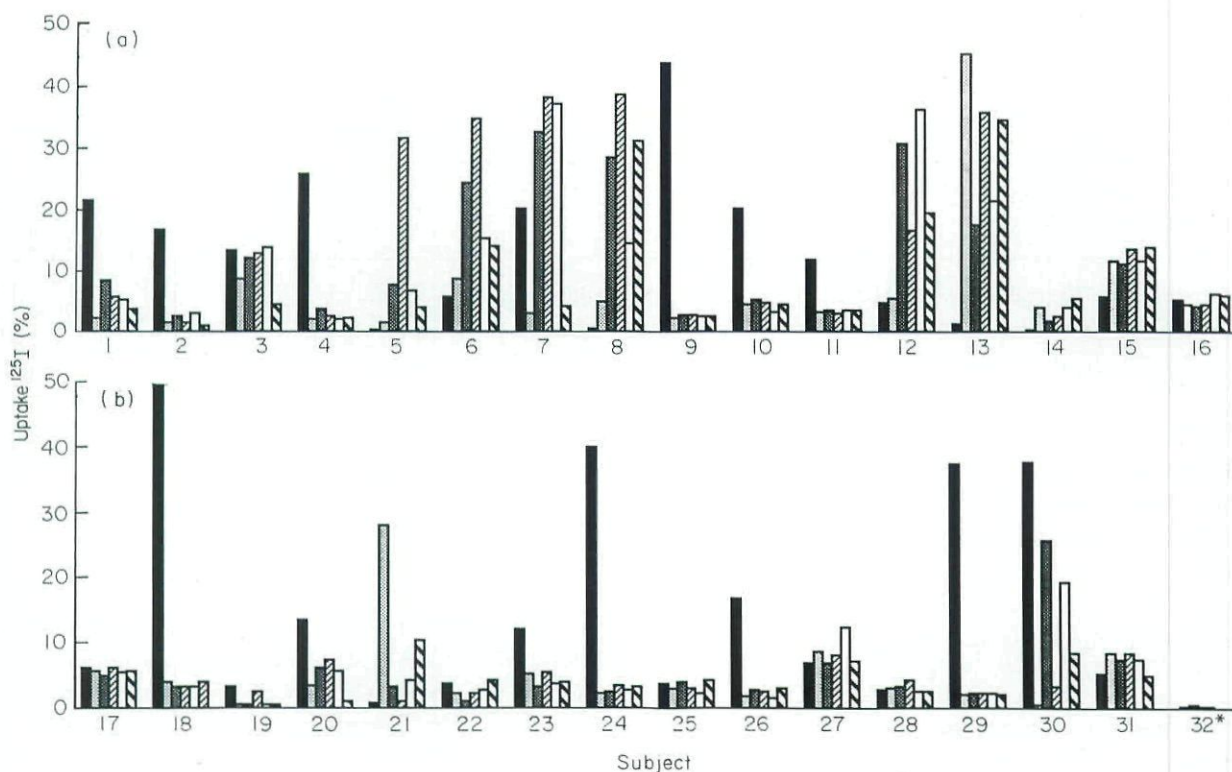


**Figure 3.** Individual IgE-activities from sera with IgE antibody reactivity to two or more *Myrmecia* venoms. ■, *Myrmecia pilosula* venom; □, *Myrmecia nigrocincta* venom; ▨, *Myrmecia tarsata* venom; ▩, *Myrmecia pyriformis* venom; ▪, *Myrmecia simillima* venom; ▤, *Myrmecia gulosa* venom. \* Serum sample no. 22 was an IgE negative control (see methods).

**Table 2.** Inhibition studies\* to detect the presence of immunological cross reaction between six different venoms of the genus *Myrmecia*

| Subject† | <i>M. pilosula</i> | <i>M. nigrocincta</i> | <i>M. tarsata</i> | <i>M. pyriformis</i> | <i>M. simillima</i> | <i>M. gulosa</i> |
|----------|--------------------|-----------------------|-------------------|----------------------|---------------------|------------------|
| A        | 86                 | 0                     | 1                 | 0                    | 0                   | 0                |
| B        | 78                 | 0                     | 0                 | 0                    | 0                   | 0                |
| C        | 51                 | 0                     | 14                | 35                   | 25                  | 0                |
| D        | 86                 | 0                     | 0                 | 37                   | 0                   | 0                |
| E        | 23                 | 24                    | 0                 | 0                    | 0                   | 46               |
| F        | 70                 | 0                     | 0                 | 4                    | 0                   | 0                |
| G        | 65                 | 0                     | 0                 | 34                   | 0                   | 12               |

\* Percentage inhibition of binding of *M. pilosula* venom (solid phase), using all six *Myrmecia* venoms as inhibitors. † Sera from *Myrmecia* ant venom-allergic subjects with IgE reactivity to two or more *Myrmecia* venoms.

venoms has no obvious pattern. It is, however, improbable that many of these individuals would have been separately stung by all six ant species because of the geographical considerations; the ants simply not being present in the allergic subjects' regions of residence or work location.

A dramatic loss of serum IgE which recognized the venoms of *M. pilosula*, *M. nigrocincta*, *M. tarsata*, *M. pyriformis*, *M. simillima* and *M. gulosa* was observed in the sera of subjects 7, 8 and 22, in serum samples taken 12 months subsequent to the initial finding of venom-reactive IgE antibodies. (Results not shown).

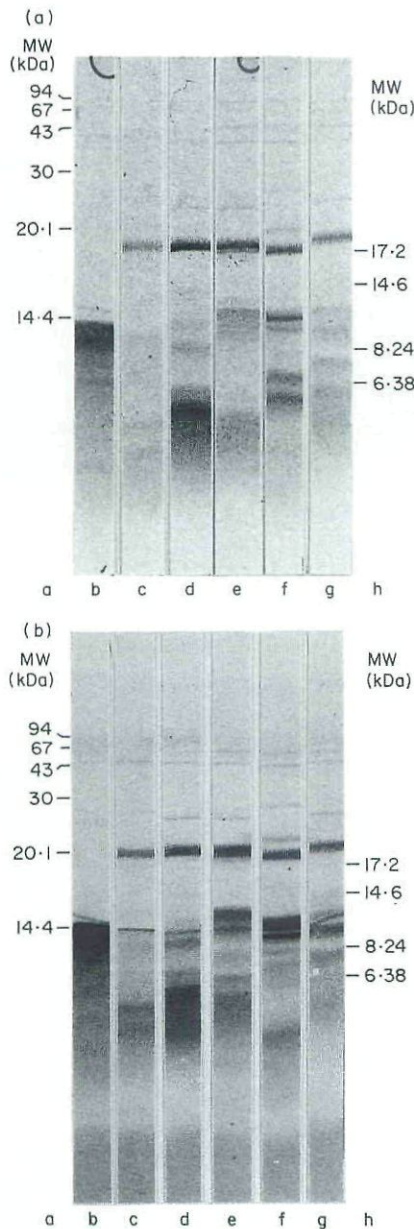


**Figure 4.** Amido black staining of six *Myrmecia* species venoms separated by SDS-PAGE. (a) Stained gel. (b) Stained nitrocellulose transfer. (a) and (h) molecular weight markers; (b) *Myrmecia pilosula*; (c) *Myrmecia nigrocincta*; (d) *Myrmecia tarsata*; (e) *Myrmecia pyriformis*; (f) *Myrmecia simillima*; (g) *Myrmecia gulosa*.

#### Specificity of IgE antibodies to *Myrmecia* venoms: Inhibition studies

A number of subjects with IgE antibodies recognizing more than one venom species, were tested in quantitative inhibition studies. In these studies *M. pilosula* venom was used on the solid phase and all six venoms were examined for inhibitory properties (Table 2). Inhibition of IgE-

binding was, in all cases, greatest with *M. pilosula*. Where inhibition was observed with the other *Myrmecia* ant venoms, no obvious pattern was discernible.

#### Electrophoretic separation of *Myrmecia* venom proteins and identification of IgE-binding components

Following SDS-PAGE and protein staining 16 protein bands were identified in the venoms of *M. pilosula*, *M. tarsata* and *M. pyriformis*. In the venoms of *M. nigrocincta*, *M. simillima* and *M. gulosa*, 14, 13 and 12 protein bands respectively, were identified (Fig. 4). The identified bands from the six *Myrmecia* venoms all migrated within the molecular weight range 2–94 kDa.

Generally, for all four staining procedures, the higher molecular weight components (i.e. > 30 kDa) were well resolved but only weakly stained. The resolution and staining intensity of the lower molecular weight components (i.e. < 30 kDa), however, varied considerably with the different staining methods. This was particularly so with the venom of *M. pilosula*. Resolution of the lower molecular weight components in the venom of *M. pilosula* was dramatically improved by the transfer of the proteins to nitrocellulose and subsequent staining with either amido black (Fig. 4b) or Ponceau S (results not shown).

Amido black was found to be the best of the four staining procedures for staining either SDS-PAGEs or nitrocellulose transfers on the basis of the clearest identification of all of the venom protein components (Fig. 4a and 4b).

Venoms from six different *Myrmecia* species were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoprobed with individual sera from *Myrmecia* venom-allergic subjects (Table 1 and Fig. 3), identified by RAST. Four IgE binding components were found in the venom of *M. gulosa*, five in the venoms of *M. pilosula*, *M. nigrocincta*, *M. tarsata*, *M. pyriformis*, and six in *M. simillima* venom. All IgE binding components ranged in size from 2 to 25 kDa (Fig. 5). In the venoms of *M. nigrocincta*, *M. tarsata*, *M. pyriformis*, *M. simillima* and *M. gulosa* the largest IgE binding component has a molecular weight 24–25 kDa but an allergen of this size is not present in the venom of *M. pilosula* (Fig 4a and 4b), where the largest IgE-binding component has a molecular weight of approximately 8.5 kDa (Fig. 5). Table 3 lists side-by-side the number and approximate molecular weights of all of the IgE-binding components identified by protein blotting in the six different *Myrmecia* venoms.

#### Discussion

Electrophoretic separation of venom proteins and their subsequent protein staining identified multiple venom

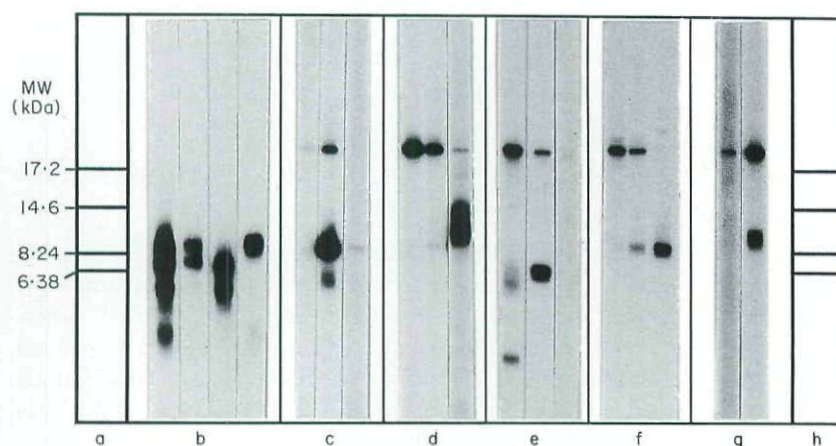


**Figure 5.** Immunoblots of IgE-binding components in the venom of six different *Myrmecia* species. (a) and (h) molecular weight markers; (b) *Myrmecia pilosula*; (c) *Myrmecia nigrocincta*; (d) *Myrmecia tarsata*; (e) *Myrmecia pyriformis*; (f) *Myrmecia simillima*; (g) *Myrmecia gulosa*. Bound IgE was detected with  $^{125}\text{I}$ -anti-human IgE and autoradiography.

**Table 3.** The apparent molecular weights (kDa)\* of the IgE-binding components† in six different *Myrmecia* venoms

| <i>M. pilosula</i> | <i>M. nigrocincta</i> | <i>M. tarsata</i> | <i>M. pyriformis</i> | <i>M. simillima</i> | <i>M. gulosa</i> |
|--------------------|-----------------------|-------------------|----------------------|---------------------|------------------|
| 8.5                | 24.0                  | 24.5              | 24.0                 | 25.0                | 24.0             |
| 7.5                | 9.1                   | 19.0              | 8.4                  | 23.0                | 18.0             |
| 5.5                | 8.7                   | 12.5              | 7.4                  | 18.5                | 12.8             |
| 4.0                | 8.2                   | 10.4              | 5.2                  | 12.4                | 10.8             |
| 2.0                | 6.2                   | 8.9               | 3.7                  | 9.6                 |                  |
|                    |                       |                   |                      | 3.8                 |                  |

\* Polypeptide markers, used to determine the apparent molecular weights of the IgE-binding molecules, † IgE-binding components separated by SDS-PAGE under reducing conditions. Transferred to nitrocellulose and immunoprobed with sera from ant venom allergic subjects.

components in each *Myrmecia* species. Up to six components from each venom also bound human IgE antibodies. Previous attempts have been made to identify the biologically-active components of ant venoms, although with only limited success [19,20]. Venoms from ants of the genus *Myrmecia* have been found to contain hyaluronidase and kinin-like activities, a non-histamine smooth muscle stimulant, histamine releasing activity [19] and a heat-sensitive haemolytic factor [20]. Histamine has also been found in these venoms in varying levels from 0.9% in *M. pilosula* venom [20] to 2% in *M. gulosa* and *M. pyriformis* venoms [19].

Biochemical activities such as phospholipase, hyaluronidase and acid phosphatase activities, together with pharmacologically active amines histamine and acetylcholine, reported in the venoms of other Hymenoptera, are reviewed by Schmidt [14].

Fatalities from stings of *Myrmecia* ants, due to the venoms' pharmacological effects, have not been reported. However, the range and severity of allergic responses,

including life-threatening anaphylaxis, is causing concern in an increasing number of allergic individuals [1,2]. Multiple IgE-binding components in the molecular weight range 2–25 kDa have now been identified in the venoms of six *Myrmecia* species. One of these components, the allergen *Myr p I* in the venom of *M. pilosula*, has been identified, cloned and characterized [15]. No similarities with other proteins, in biochemical activity or protein sequence homology, was found [15]. Other IgE-binding components in the six *Myrmecia* venoms, remain to be characterized and studied further.

Taxonomic relationships between *Myrmecia* species have been established using a range of factors including morphological, genetic and behavioural characteristics [4]. These relationships suggest that the species of *M. gulosa*, *M. pyriformis*, *M. simillima* and *M. tarsata* are closely related, while *M. nigrocincta* and *M. pilosula* are more distant relatives [4]. Although similarities in molecular weight exist between the IgE-binding components in the different venoms (Table 3), it has yet to be

determined if these are related and/or crossreacting IgE-binding molecules. As *Myrmecia* species have a wide distribution throughout Australia, the possibility of individual contact with more than one, and up to six of the species studied here is unlikely. Therefore, the presence, in some subjects, of antibodies to all six venoms may be a reflection of true immunological crossreactivity. Currently, investigations are being undertaken to pursue this question of allergenic crossreactivity between venoms from species within the genus *Myrmecia*.

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