PART I: INTRODUCTION

Chapter 1: Literature review

The literature on stinging insect allergy is reviewed in this chapter to provide the necessary information for planning and interpreting clinical research into the efficacy and application of immunotherapy for allergy to Australian stinging ants. To begin with, the stinging insects found in Australia will be examined. Knowledge of their distribution and distinguishing features is required to enable clinical information, diagnostic skin testing and serum IgE analysis to be placed in proper perspective. Special attention will be paid to the species found in Tasmania, where this research was performed.

1.1 Stinging insects of medical importance in Australia

Within the order Hymenoptera ("membrane-winged insects"), those with an ovipositor (egg-laying device) adapted as a weapon to deliver venom are classified within the legion Aculeata. Although many species can deliver potentially allergenic venom, repeated stings and thus allergic reactions are generally confined to those that form large colonies. These "social insects" are characterised by a dominant female and specialised female workers, and fall within the families Apidae (bees), Vespidae (social wasps) and Formicidae (ants), all of which are represented in Australia.

Several biting insects not classified within the Aculeata such as ticks, march flies and mosquitoes have been associated with anaphylactic reactions, presumably to salivary substances.[4] Several stingless ants may trigger allergic reactions when the contents of their abdominal venom glands are sprayed into a bite, although these reactions are almost always mild.[5,6] As this thesis focuses on stinging insect venom allergy, biting insects will not be discussed further.

The propensity for stinging insects to cause anaphylaxis depends not only on regular contact with humans by virtue of colony size/numbers, but also on aggressive tendencies &/or a relationship with human dwellings or agriculture. Therefore, Aculeates outside the abovementioned families that are solitary or form small social groups have also been reported to cause anaphylaxis, and conversely many social Aculeates capable of stinging have not yet been associated with human anaphylaxis.

1.1.1 Phylogeny of the Aculeata

For those Aculeates reported in the medical literature to pose health threats due to anaphylactic reactions, likely evolutionary relationships (phylogeny) according to

the recent work of D. J. Brothers,[7] are presented in Figure 1.1. An appreciation of phylogeny may help to predict the potential for venom allergen similarities and thus human IgE antibody cross-reactivity.

The term "wasp" refers to numerous social and solitary insects within the Apoidea (sphecid wasp and bee) and Vespoidea (wasp and ant) superfamilies. Wasps are characterised by slender bodies, narrow waists and a well-developed abdominal stinger. They usually feed on other insects (including agricultural pests), spiders and decaying animal matter. It is generally accepted that bees and ants both arose from wasp-like ancestors, with ants and social wasps having closer evolutionary relationships to each other than they do to bees or the solitary sphecid wasps. However, phylogenetic relationships are constantly subject to revision, particularly with the application of new molecular biology techniques to support morphological and behavioural analyses. Aculeate relationships are no exception, and several areas of uncertainty remain.[7,8]

1.1.2 Family Apidae

In contrast to wasps, bee mouthpieces are adapted for collecting nectar from flowers. Bee larvae feed almost exclusively on nectar and pollen, rather than insect or spider prey as is the case for wasps.

1.1.2.1 Native bees

Most native bee species worldwide are solitary or form small social groups without specialisation (that is, without differentiation into queens, drones and female workers). Because they do not form large colonies and do not aggressively guard their nests, the majority of bee species do not commonly encounter humans. However, on hot days they may be attracted to lap up perspiration, and are thus commonly referred to as "sweat bees". During this activity if caught in clothing or crushed, a female sweat bee will deliver a sting. Although the Australian continent has an abundance of native bee species, only a few cases of anaphylaxis and one death from South Australia have been reported following presumed *Lasioglossum* spp. stings.[9]

1.1.2.2 Honeybee

Apis mellifera (the domesticated honeybee) (Figure 1.2A) is the sole member of the genus *Apis* and is cultured worldwide to produce honey and for agricultural pollination. Colonies are large and well guarded. Shear numbers and a close association with human activities result in a large number of human encounters. The honeybee stinging apparatus is unique amongst the Aculeata, in that the sting is barbed and remains in

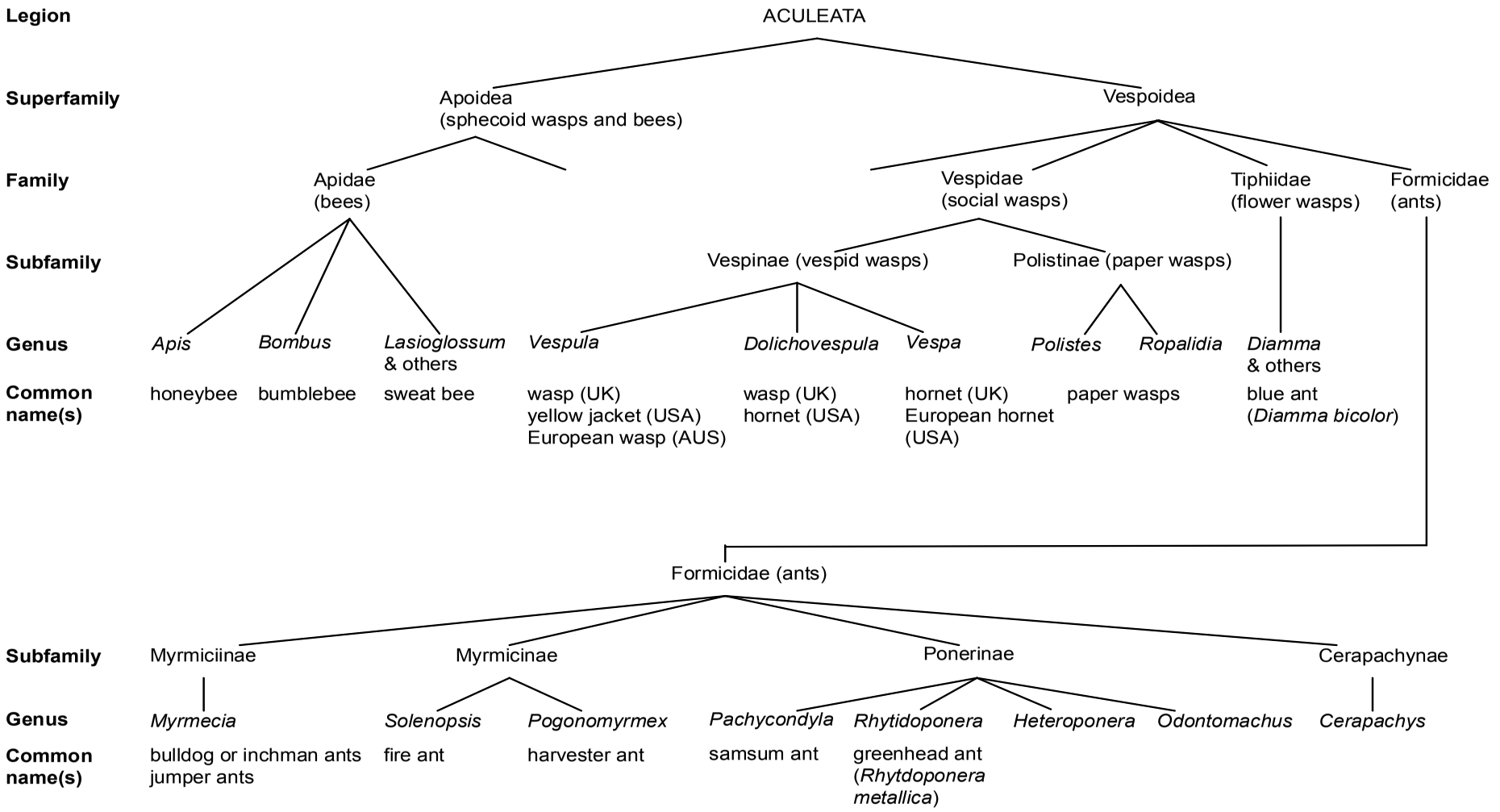


Figure 1.1: Phylogeny of the Aculeata (stinging Hymenoptera)

the skin. The venom sac detaches from the insect's body and continues to contract and inject venom.

1.1.2.3 Bumblebees

Members of the genus *Bombus* (bumblebee, Figure 1.2B) form small colonies and are considered less aggressive than honeybees, with allergy reported to be rare. However, since the mid 1980's their commercial use has become widespread. Consequently, sting exposure and thus specific bumblebee venom allergy, particularly as a work-related phenomenon, may be on the increase.[10] Bumblebees have been introduced to Tasmania and are now prolific through much of the state (personal observation), but have not yet been introduced to mainland Australia.

1.1.3 Family Vespidae

This family is represented on most landmasses. Vespidae can be distinguished morphologically from honeybee by their smooth body surfaces and narrow waists. Vespinae (vespid wasps) are distinguished from Polistinae (paper wasps) by a square-shaped abdomen that abruptly narrows at the waist (Figure 1.2C-D).

Common names for the major genera of the vespid wasp family (Vespinae) are somewhat confused between continents. *Vespula* spp. are termed "yellow jacket" in the USA, and "wasp" in the UK. The slightly larger *Dolichovespula* spp. are termed "wasp" in the UK and "hornet" in the USA. The much larger *Vespa* spp. are termed "hornet" in the UK and "European hornet" in the USA. In the USA, the term "wasp" is reserved for paper wasps.[11]

In this thesis, the terms "wasp" will be used to encompass all members of the family Vespidae, and the terms "paper wasp" and "vespid wasp" (or "vespid") will be used to refer to the subfamilies Polistinae and Vespinae respectively.

1.1.3.1 Paper wasps

Australia has some 35 native species of paper wasp in the genera *Polistes* and *Ropalidia* (Figure 1.2C). They build their nests hanging from eaves of houses or tree branches or in hollow trees and fallen logs. Paper wasps feed mainly on caterpillars and are therefore useful for pest-control in agricultural areas. They attack animals and humans passing near their nests in defence, but stings are said to be relatively uncommon away from the nest. Although found throughout mainland Australia, the greatest concentration and variety of species is found in Queensland. Paper wasps have not been found in Tasmania.

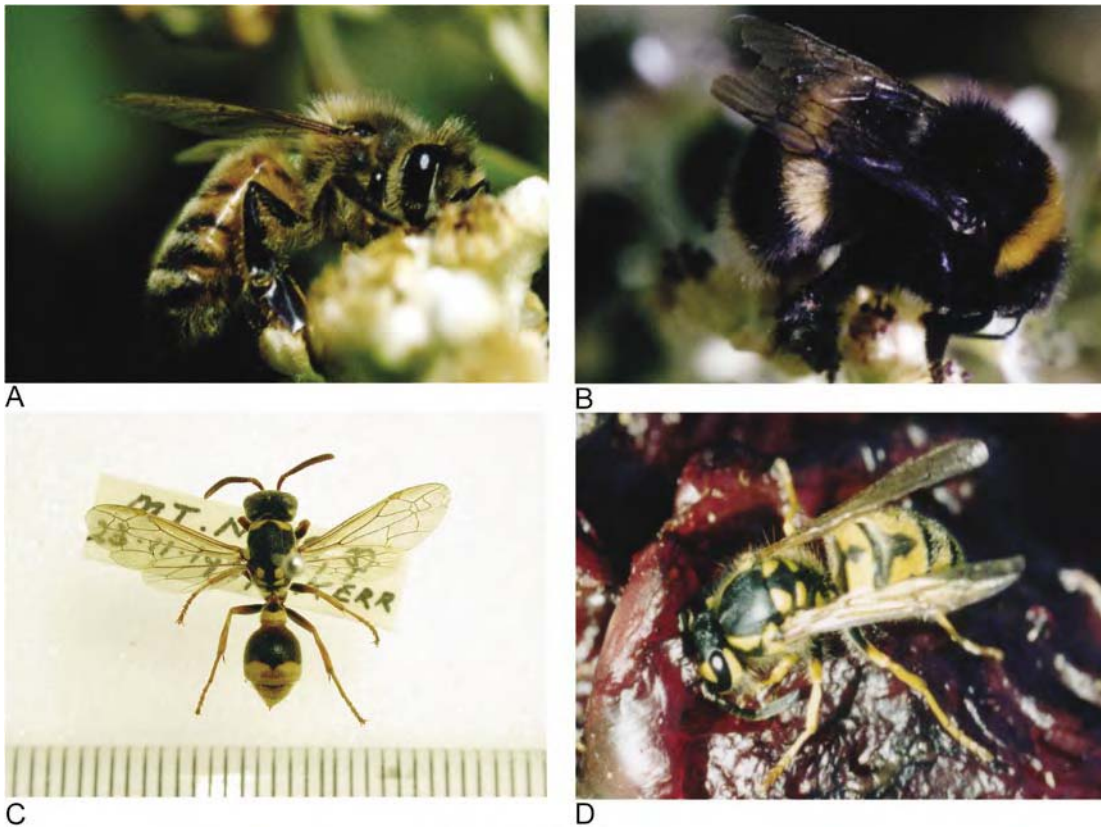


Figure 1.2: Bees and wasps: **A-** Honeybee, *Apis mellifera*; **B-** Bumblebee, *Bombus* spp; **C-** Native paper wasp, *Ropalidia gregaria*; **D-** European wasp (yellow jacket), *Vespula germanica*. Photograph C courtesy of Dr Ken Walker, Department of Entomology, Museum Victoria.

1.1.3.2 Vespid wasps

Mature vespid nests are very large, and mount vigorous attacks if disturbed. Accidental stings are said to be more common away from the nest than is the case for paper wasps, attributed to their wider feeding tastes, including meat and sugary foods. A number of deaths have been reported following upper airway stings when vespids feeding on sweet drinks have been accidentally swallowed.

There are no native Australian vespids, but *Vespula germanica*, known locally as the European wasp or German wasp (Figure 1.2D) has been introduced. Established first in Tasmania then Victoria, it is now found in all mainland states despite attempts at eradication. *Vespula vulgaris*, sometimes called the "English" wasp, but indistinguishable from *V germanica* to the layperson, has also been found in Victoria.

1.1.4 Other Vespoidea families

There are another seven families, mainly solitary wasp species, within the superfamily Vespoidea. Although most females are capable of delivering potentially allergenic

venom to humans only one, a native Australian wingless ground-dwelling "flower wasp", *Diamma bicolor*, has been reported in the medical literature to cause anaphylaxis.[1] This large insect, found in south-eastern Australia and Tasmania and known commonly as the "blue ant" or "bluebottle ant", sometimes nests in domestic lawns and is likely to emerge when the lawn is watered, delivering a painful sting if accidentally stepped on.

1.1.5 Family Formicidae (ants)

Some 10,000 species of ant are currently divided into 16 modern subfamilies and 4 extinct fossil subfamilies, however there is significant uncertainty with regard to phylogeny and thus classification.[7] Representatives of several subfamilies have been reported to cause anaphylaxis. In many, the stinger has become non-functional (for example the subfamily Formicinae) yet painful bites and, very rarely, mild systemic allergic reactions, may occur when an ant sprays formic acid and other substances into the wound.[5,6] The following discussion will be limited to stinging ants.

Unless otherwise noted, the following information on Australian species and distributions has been taken from the Commonwealth Science and Industrial Research Organisation (CSIRO) resource "Australian Ants Online".[12]

1.1.5.1 Subfamily Myrmicinae

Imported fire ants (IFA) (*Solenopsis* spp.) are a significant cause of anaphylaxis in the Americas. Although Australia has a number of native *Solenopsis* species, none of these have been reported to cause anaphylaxis. *Solenopsis invicta* (the "red fire ant") has recently become established in Australia, with the first recorded case of anaphylaxis to its sting leading to a warning of dire ecological consequences if further spread is not contained.[13,14]

Another member of the subfamily Myrmicinae, the "harvester ant" (*Pogonomyrmex* spp.) has also been reported to cause anaphylaxis in North America.[15] No members of the genus *Pogonomyrmex* are found in Australia.

1.1.5.2 Subfamilies Ponerinae and Cerapachynae

Worldwide, four genera of the subfamily Ponerinae have been reported to cause sting anaphylaxis; *Pachycondyla*, *Heteroponera*, *Odontomachus*, and *Rhytidoponera*. *Pachycondyla chinensis* is found in Korea, Japan, and China, with clusters of anaphylaxis reported in the medical literature from heavily infested areas of Korea,[16-18] and isolated cases from Japan.[19] In the United Emirates, *Pachycondyla sennaarensis*,

known as the "samsum ant", has been recognised as a significant public health threat.[20,21] From Chile, anaphylaxis to *Heteroponera carinifrons* venom has been reported.[22] A single case of relatively mild anaphylaxis to *Odontomachus bauri* (a genus which is common in tropical regions around the world) has been reported from Venezuela, the authors noting that it is not a particularly aggressive ant unless its nest is disturbed.[23]

The subfamily Ponerinae (including several *Pachycondyla*, *Odontomachus* and *Heteroponera* species) is well represented throughout Australia, but anaphylaxis to these genera appears to be uncommon. Anaphylaxis to the "greenhead ant" *Rhytidoponera metallica* (Figure 1.3), found in many areas of mainland Australia, has been reported from Queensland.[4] A passing reference incriminating *Pachycondyla mayri* (previously *Bothroponera mayriem*), an inhabitant of coastal Queensland, as a cause of anaphylaxis has also been made.[1] Although no clinical details have been published, native *Odontomachus*, *Pachycondyla* (*Brachyponera*) and *Cerapachys* species (Subfamily Cerapachynae) have been claimed to cause anaphylaxis in Queensland.[13] This claim was based on ants sent for identification, apparently after having caused allergic reactions; however there has been no expert clinical verification (Cas Vanderwoude, Fire Ant Control Centre, Queensland; personal communication).

1.1.5.3 Subfamily Myrmiciinae

The *Myrmecia* are the sole living genus of the subfamily Myrmeciinae, and found only on the Australian continent and surrounding islands. Considered one of the most

Figure 1.3: *Rhytidoponera metallica*. Photograph courtesy of Hirotami T. Imai, the Japanese ant database group (JADG)

ancient groups of ants and thus a "living fossil" of great interest to biologists,[24,25] it is also one of the most karyologically diverse animal genera.[26] Of 89 currently named species, 88 are endemic to Australia and one (*Myrmecia apicalis*) to New Caledonia. An introduced population (*Myrmecia brevinoda*) has also been reported from New Zealand.[26] *Myrmecia* are distinguished by their large size (10-25mm), well-developed anteriorly positioned eyes, and long, strongly toothed and forwardly directed mandibles. They are considered a "remnant" species; their habitat having been gradually confined to the cooler and relatively moist southeastern coastal and mountain areas as the Australian climate and vegetation has changed.

A review of named species by K. Ogata and R.W Taylor, yet to be superseded, divides the *Myrmecia* into 9 species groups.[26] From a clinical perspective, the most important –that is, encountered frequently by humans– appear to be those in the pilosula, nigrocincta and gulosa species groups.[1,2,27,28] These groupings are based largely on morphological comparisons; little is known about their genetic relationships and Ogata and Taylor have cautioned that this system should be viewed as preliminary.

Species-level identification of *Myrmecia* requires a detailed assessment of morphological characteristics, as external colouration is frequently misleading. "Mullerian mimicry", whereby one dangerous insect evolves to looks like another, is said to be a form of "collective advertising" to potential predators (as compared to Batesian mimicry where a harmless insect looks like a dangerous one to ward off predators). This may result in different species taking on similar colouration.[24] Thus, the general descriptions given here should be used with caution. Accurate species identification is required in an area where multiple species coexist; reference to identification charts[12,26] and the assistance of an experienced entomologist are required.

Unless otherwise referenced, the following descriptions and notes on geographical distribution have been taken from Ogata and Taylor.[26] Only species whose venoms have been investigated against allergic patient sera,[27] &/or reported to be clinically significant,[1,2,25,28] &/or encountered frequently in Tasmania (personal observation from field trips) will be described.

The pilosula and nigrocincta groups (the "jumper ants")

Previously thought to be a single species, *Myrmecia pilosula* (jack jumper ant, JJA) (Figure 1.4A) is in fact a karyologically diverse species complex, consisting of several sibling species.[29,30] Specimens have been collected from the Murray-Darling basin and

southeastern coastal and highland regions from South Australia through to southern Queensland, and from Tasmania and the southern tip of Western Australia.

M pilosula is characterised by its size (smaller than most other *Myrmecia*, at 10-12 mm), colouration (jet black expect for yellow-orange mandibles and leg tips), and jumping movements that are reminiscent of "jumping jack" firecrackers. It is also known as the "jumper ant" in Victoria and the "hopper ant" in South Australia, but these names also include other species (see below). Some laypersons and medical staff also call *M pilosula* a "bull ant", along with other members of the genus (personal observations). *M pilosula* is notoriously aggressive, described by one author as "apparently fearless".[1] A daylight forager, it has well developed vision and is attracted to movement, leading to frequent human encounters.

Myrmecia nigrocincta (Figure 1.4B) is relatively small (10-15mm) with characteristic alternating black and orange-red body colouration. It is found in the same distribution as *M gulosa*. *M nigrocincta* is also commonly referred to as a "jumper ant", displaying movements identical to *M pilosula*. Indeed, it has previously been classified with *M pilosula* in a separate genus (*Promyrmecia*).[1]

Myrmecia chasei has been found only in southwest coastal WA. It is similar to *M pilosula* in size and hopping movements (personal observation), and in colouration closely resembles *M nigrocincta*. It has been reported to the CSIRO as being responsible for cases of anaphylaxis in areas "near Perth".[25]

The gulosa group

Gulosa species group ants are generally large (15-22 mm long) and known commonly as "bulldog" or "bull" ants on mainland Australia, in reference to their impressive mandibles. In Tasmania, they are generally referred to as "inchman" or "inch" ants as befits their size.

Myrmecia pyriformis and *Myrmecia forficata* (Figure 1.4C) are similar in appearance and coloration (dark brown body with black gaster), being easily confused with one another. They are found mainly in the southeastern coastal, highland and Murray-Darling basin regions of mainland Australia; Victoria and New South Wales and Australian Capital Territory. *M forficata* is found throughout Tasmania, where it is one of only three *Myrmecia* (*pilosula*, *esuriens* and *forficata*) to be found in any appreciable numbers.

Myrmecia esuriens, characterised by bright orange mandibles, legs and postpetiole (Figure 1.4D) is endemic to Tasmania and its colouration, a possible example of Mullerian mimicry, may lead it to be confused by laypersons as a very large JJA. However, it is less frequently encountered than other Tasmanian *Myrmecia* and is relatively timid (personal observations). It is thus an infrequent cause of stings.

Myrmecia gulosa was one of the first Australian animals to be taxonomically described by Joseph Banks or Daniel Solander of James Cook's Endeavour (1770). It has a distinctive orange-red body and black-tipped gaster (Figure 1.4E), and is found along the southeastern and northeastern coastal, highland and Murray-Darling basin regions of mainland Australia; Victoria, New South Wales, Australian Capital Territory and Queensland.

Myrmecia simillima (Figure 1.4F) has an all black body with light orange mandibles and antennae. It is found in the same areas as *M gulosa*. *Myrmecia tarsata* (Figure 1.4G) has a green-black colouration apart from a yellow tipped gaster. It has a similar distribution to *M gulosa*, although it has not been found in the Murray-Darling basin, and is found in South Australia. *Myrmecia rufinodis* (Figure 1.4H) is found only in the Southern Gulfs - South Australian region. It has been reported to the CSIRO as being responsible for cases of anaphylaxis in areas around Adelaide,[25] although anecdotal evidence suggests *M pilosula* is a far greater problem in that region (Dr Robert J Heddle, personal communication).

1.2 Insect sting anaphylaxis

1.2.1 Clinical presentation

The potential for life-threatening reactions and death following insect sting has been known since antiquity and recorded in the English-language scientific literature as early as 1836.[31] In 1902 Portier and Richet first coined the term "anaphylaxis", derived from the Greek a- (contrary to) and phylaxis (protection) after observing lethal shock reactions upon repeated exposure of dogs to sea anemone toxin whilst attempting to induce immunity to that toxin's physiological effects.[32] Although this phenomenon had been recorded previously, Portier and Richet were the first to recognise its significance, laying the foundation for further research into the nature of anaphylaxis, including insect sting reactions.

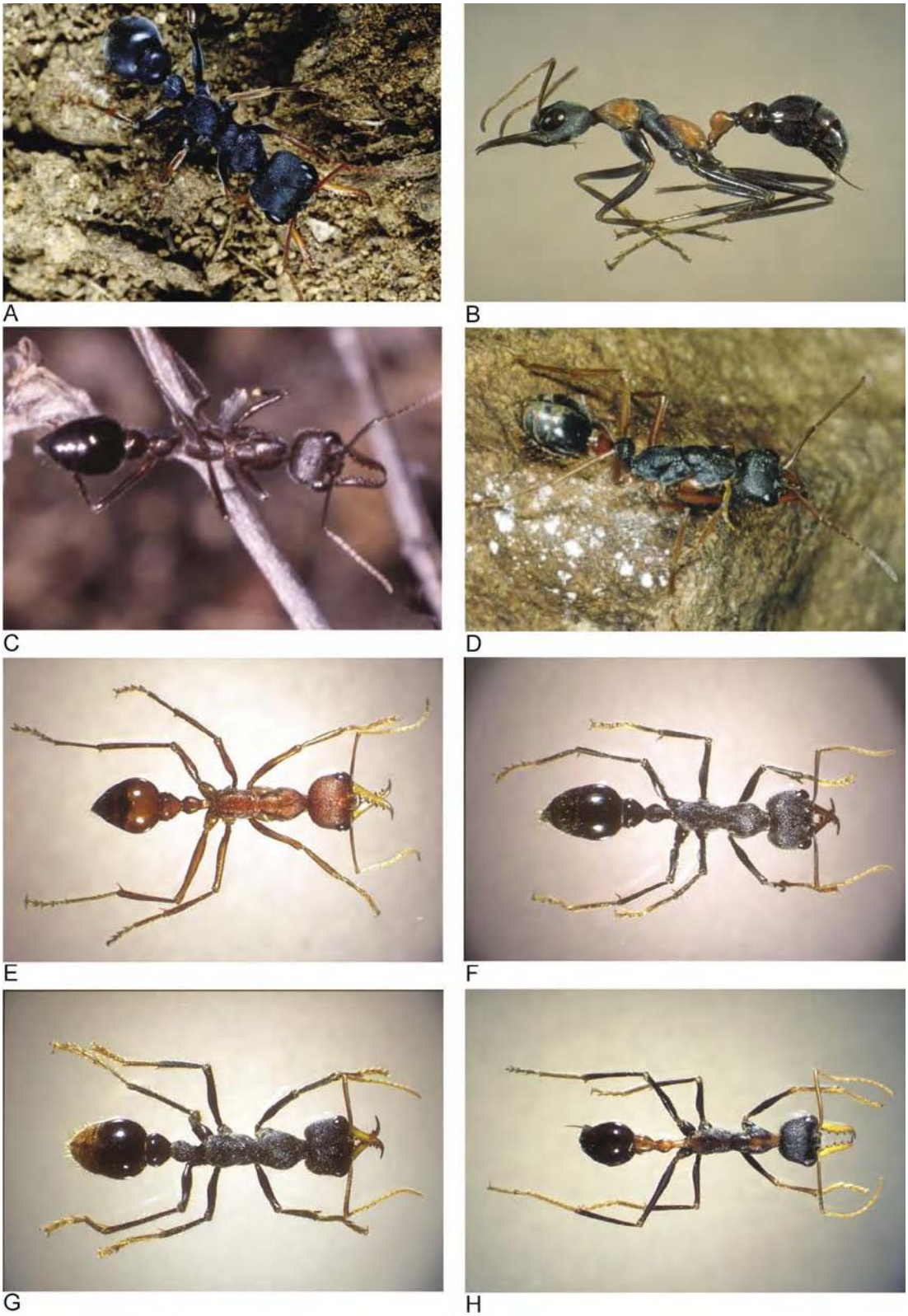


Figure 1.4: Selected ants of the genus *Myrmecia*. (i) **Jumper ants** are 10-12mm in length; **A-** *Myrmecia pilosula* ("jack jumper"); **B-** *Myrmecia nigrocincta*. (ii) The larger **"bulldog" or "inchman" ants** range from 15-25 mm in length; **C-** *Myrmecia forficata*; **D-** *Myrmecia esuriens*; **E-** *Myrmecia gulosa*; **F-** *Myrmecia simillima*; **G-** *Myrmecia tarsata*; **H-** *Myrmecia rufinodis*. Plates B, E, F, G and H courtesy of Hirotami T. Imai, the Japanese ant database group (JADG).

1.2.1.1 Typical immediate reactions

A.T. Waterhouse recognised severe honeybee sting reactions as a form of anaphylactic shock in 1914, describing a typical systemic reaction in a 53 year old man as follows: [33]

> *...while walking in the garden of St John's College, Oxford, he was stung by a bee on the upper margin of the right ear. Remembering his former experience he left the college, and by the time he had reached the street; he began to feel a constriction of the throat and difficulty in breathing. He walked across the road to the nearest chemist's shop and collapsed on the floor. When I saw him, about ten minutes later, he was semi-conscious and restless, with sighing respirations. The right side of the face was congested and slightly swollen. He was sweating, and the extremities were pale, cold, and clammy. The pulse could not be felt at the wrist and the heart sounds were very feeble. The sting had been extracted and ammonia had been applied to the ear before my arrival. He was given some sal volatile and compound tincture of cardamoms and 5 minims of liquor strychnine were injected under the skin of the arm. He gradually recovered full consciousness, and in about three-quarters of an hour from the time when I first saw him he was removed to his lodgings. His pulse could now be felt. His condition improved steadily, and on the following day his heart was acting normally. He had no diarrhoea and did not vomit. He was very listless for a few days, but made a good recovery.*

Although many authors have reserved the term "anaphylaxis" for severe reactions, the same mechanisms trigger mild reactions so the term is sometimes applied to generalised immediate-type hypersensitivity reactions of any severity. Clinical features may include one or more of the following: diffuse skin reactions (erythema, itch, urticaria), gastrointestinal features (epigastric discomfort, abdominal pain, nausea, vomiting, diarrhoea), facial angioedema, pharyngeal &/or laryngeal oedema (difficulty speaking or swallowing, upper airway obstruction) respiratory involvement (chest tightness, breathlessness, bronchospasm, hypoxia), hypotension or symptoms suspicious for hypotension (light-headedness, dizziness, weakness, collapse from the upright position), and outright cardiovascular collapse (shock, unconsciousness, incontinence of urine &/or faeces, cardiac arrest). Other features may include nasal congestion and watery eyes, throbbing vascular headache, malaise, anxiety, and a feeling of impending doom.[34,35]

It is difficult to determine the frequencies of individual reaction characteristics from most published studies because data is presented in a condensed form using various grading systems, and because of the recollection biases inherent in such studies. A tabulation of the relative frequencies of individual symptoms reported from one series of 400 people assessed after experiencing a generalised reaction to a single honeybee or wasp sting is presented in Table 1.1. Although the authors claim this to have been an "unselected" group, the method of recruitment is not stated. Twenty-nine percent

Table 1.1: Frequency of individual reaction features from a series of 400 bee and wasp sting systemic reactions[34]

Feature		Feature	
Generalised swelling	56%	Violent throbbing headache	12%
Generalised itch	55%	Asthma	8%
Urticaria	49%	Dysphagia (swelling of the throat)	8%
Dyspnoea	43%	Chills or fever or both	7%
Weakness	39%	Dizziness	7%
Decrease in blood pressure	37%	Incontinence	6%
Loss of consciousness	29%	Non-urticarial eruption	3%
Tachycardia	22%	Feeling of faintness	3%
Nausea	20%	Acute rhinitis	2%
Vomiting	14%	Abdominal cramps	2%
Anxiety	14%	Swelling of the tongue	2%

experienced loss of consciousness, and hypotension was recorded in 37%, although their definition of hypotension was not provided. In other studies, 11-26% of cases have been described as severe (including unconsciousness and hypotension),[35-38] however direct comparisons between studies are difficult due to the use of different grading systems and differing populations (eg adult versus paediatric).

1.2.1.2 Cardiac manifestations of anaphylaxis

Tachycardia immediately following a sting is typical,[39,40] and may be due to anaphylactic mediators, pain, anxiety or a compensatory response to peripheral vasodilation. Other, less common cardiac effects during sting anaphylaxis, observed in otherwise healthy people, have included relative bradycardia (falling heart rate despite worsening hypotension),[39,40] profound but eventually reversible myocardial depression without any evidence of underlying heart disease and associated with non-specific ST changes,[41] reversible ECG changes indicating myocardial ischaemia,[39,42] and classical angina pectoris associated with ECG changes whilst hypotensive.[40] Severe reversible myocardial ischaemia causing left ventricular failure and pulmonary oedema has been reported as due to sting anaphylaxis in a man with underlying ischaemic heart disease,[43] and completed myocardial infarctions have occurred in patients suffering marked hypotensive reactions.[44] Although not reported during sting anaphylaxis, ventricular fibrillation has been reported rarely (4 out of 186 cases) when anaphylaxis has occurred under anaesthesia, although may have been due to increased ventricular excitability induced by the combination of halothane and adrenaline.[45]

1.2.1.3 Neurological syndromes associated with anaphylaxis

Seizures,[46,47] transient cerebral ischaemic syndromes,[48] persistent neurological disability with hemiplegia,[49] prolonged confusional states,[47] extrapyramidal syndromes,[50] and severe generalised encephalopathy leading to death or permanent disability,[51] have been reported to be associated with insect sting anaphylaxis.

Neurological events are often associated with, and probably secondarily due to severe anaphylactic shock and reduced cerebral oxygen delivery.[42,48,51] However in some cases quite severe neurological deficits have occurred rapidly along with some features of anaphylaxis but little or no evidence of severe circulatory compromise or hypoxaemia.[46-49] Such cases have been associated with papilloedema and venous engorgement suggestive of raised intracranial pressure,[47] and cerebral infarction.[49] One study has claimed that significant immediate central nervous systems occurred without any evidence of cardiovascular involvement in 24% of sting reactions, leading to the conclusion that anaphylactic reactions can effect the CNS directly causing symptoms suggestive of tissue ischaemia.[48] Neuro pathological findings from lethal cases of anaphylaxis will be discussed under 1.2.4.3 below.

1.2.1.4 Speed of reaction onset

A retrospective analyses of field stings,[35] and prospective observations during deliberate sting challenges,[52] have noted that severe (hypotensive) reactions tend to have much more rapid onset (within minutes of a sting) than for less severe reactions (up to 1 hour). Rarely, sting reactions may have a much slower onset. The reported proportions of systemic allergic reactions with delayed-onset (hours to days) range from 1.0 to 2.8%.[34,37]

1.2.1.5 Protracted and biphasic reactions

Protracted and biphasic reactions may represent clinical variants of delayed-onset anaphylaxis. Protracted anaphylaxis refers to a poor response to treatment with reaction features persisting for several hours. Biphasic anaphylaxis refers to complete resolution with initial treatment followed by a recurrence during the following 24 hours, but may result from a temporary effect of treatment rather than a truly “biphasic” reaction. Presumably, newly generated mediators and recruitment of inflammatory cells (as opposed to immediate release of stored mediators) are involved in delayed-onset, protracted and biphasic reaction patterns.

Protracted and biphasic reactions have been recognised principally in food-anaphylaxis.[53,54] Several investigators have also reported the occurrence of biphasic

reactions following insect stings, usually mild,[55] but occasionally severe.[56] There have been reports of severe protracted sting anaphylaxis requiring protracted periods of resuscitation, including intra-aortic balloon pump circulatory support.[41] The true incidence of biphasic reactions is unknown. Currently available studies report biphasic reactions in 3-28% of cases,[53,55-58] but are exclusively retrospective, involve small numbers with varying aetiology (food vs. sting vs. iatrogenic), and tend to involve mainly severe cases treated with large doses of adrenaline,[56] or admitted to a medical ward or intensive care unit.[53,57,58]

1.2.1.6 "Serum sickness"

A syndrome similar to serum sickness –delayed fever, rash, arthralgia, joint swelling and lymphadenopathy– is recognised to occur following insect sting, with or without preceding anaphylaxis.[59] An incidence of 5% has been reported by one series,[34] One of the first recorded cases of "serum sickness" also reported in addition a persistent large local reaction with fluctuating angioedema.[60] In classic serum sickness (type III hypersensitivity), circulating antigen-antibody immune complexes in response to large initial antigen loads are thought to be responsible.[61] However, the amount of antigen injected by a sting is tiny, and typical features of serum sickness, including raised venom-specific IgG antibodies, a decline in C4 and C2, circulating immune complexes and evidence of renal involvement (hematuria), have not been demonstrated.[62]

1.2.1.7 Large local reactions

Hymenoptera stings normally cause a painful, itchy red lesion with local swelling, due to a direct effect of the venom rather than hypersensitivity. However, in some individuals a large local reaction may occur, defined by one group as being >10 cm diameter and lasting for more than 24 hours.[63] Infections appear to be extremely rare, probably due to the bacteriostatic nature of hymenoptera venoms,[11] although a fungal skin infection (Sporotrichosis; *Sporothrix schenkii*) has been reported following fire ant sting.[64] Some deaths may occur in people without systemic allergy where a sting occurs to the upper airway, causing local oedema, airway obstruction and thus asphyxiation.[65-67]

1.2.1.8 Other syndromes

Rare neurological syndromes have been associated with single insect stings, but may be coincidental. Supposed associations have included reversible motor &/or sensory neuropathies nearby to the original sting site,[46,68,69] reversible optic nerve dysfunction with associated papilloedema following a sting to the temple.[69] A fulminant and relapsing disease leading to death has also been reported, characterised by widespread

demyelination of both central and peripheral nervous systems and necrosis of brain and spinal cord and attributed to a delayed hypersensitivity reaction.[70] Investigators have also attempted to link cases of myasthenia gravis, Guillain-Barre syndrome and multiple sclerosis to insect stings.[59,71-73]

Other syndromes that have been associated with honeybee or wasp stings have a included a severe vasculitis resembling Henoch-Schonlein Purpura,[74,75] thrombocytopenia,[76,77] haemolytic anaemia,[78] and kidney damage unrelated to rhabdomyolysis or shock.[79] Again, it must be recognised that coincidence is a likely explanation.

1.2.2 Pathophysiology

1.2.2.1 Animal models and the concept of anaphylactic "shock organs"

Early animal studies of the pathophysiology of anaphylaxis found organ system involvement to vary between animal models, with the major organ involved being termed the "shock organ" for that particular species.[80] This concept has been repeated as recently as 2002.[81] In the guinea pig intense bronchospasm leading to hypoxia was considered the principal cause of death,[80,82] leading to secondary cardiac ECG abnormalities comparable to those caused by asphyxia.[83] In rabbits, severe vasoconstriction in the pulmonary arterial system appeared to cause hypoxia and circulatory collapse, and again cardiac ECG abnormalities have been considered to be secondary to asphyxia.[83] In dogs, severe hepatic congestion was considered the major cause of lethal shock, whilst in rats and mice, vascular collapse appeared to the main pathophysiologic feature leading to death.[80]

Contrary to the above concepts, the manifestations of guinea pig anaphylaxis have been shown to include, in addition to intense bronchospasm, an initial sinus tachycardia followed by progressive impairment of atrioventricular conduction leading to complete block, increased ventricular automaticity (including ventricular fibrillation) and cardiac contractile failure. These also occur in an isolated guinea pig heart model, indicating these features to occur in the absence of hypoxia. Furthermore, rapid and profound falls in systemic blood pressure occur with the sinus tachycardia phase (the phase associated with sustained cardiac output in isolated heart experiments), suggesting systemic vasodilation,[84] and severe coronary vasospasm also appears to be a consistent feature.[85]

In the canine model, severe systemic vasodilation,[80] increased pulmonary vascular resistance and cardiac contractile failure occur in addition to liver engorgement,[86] and in the rabbit model, cardiac anaphylaxis appears to occur concurrently with pulmonary

vasospasm.[80] Pulmonary vasospasm has also been demonstrated in a monkey model,[87] and subsequent work has demonstrated that in some monkeys, cardiovascular collapse can be due entirely to decreased preload and pulmonary hypertension whereas in others the decrease in cardiac output is more severe and probably associated with reduced myocardial contractility.[88]

Another complicating factor in the interpretation of animal models relates to methods of sensitisation and subsequent exposure. Sensitisation is either passive by injection of hyperimmune serum from another animal, or active by subcutaneous or intra peritoneal injection of antigen. Reactive antibodies transferred by passive sensitisation may be predominantly of the IgG class, whereas active sensitisation tends to results in an IgE response. Both antibody classes can mediate anaphylaxis in animal models. IgG-mediated reactions were postulated to be more likely to display myocardial depression by one group.[89,90] The mode of exposure triggering anaphylaxis once sensitisation has been achieved is also important. Auer and Lewis, with their initial description of guinea pig anaphylaxis found the clinical syndrome to be dominated by bronchospasm and hypoxia following intravenous injection of antigen. However, different clinical features, more suggestive of circulatory collapse, occurred with subcutaneous injection:[82]

> *"If the intoxicating dose be given subcutaneously in animals ordinarily hypersensitive, the course of the intoxication is prolonged and its symptoms are quite different. In a most extreme example, the animal becomes sick in about an hour after the injection, and dies in from four to six hours, or after the sixth to the twelfth hour shows distinct and finally rapid recovery. In this type of reaction the respiratory convulsions may be quite lacking. The animal's coat becomes rough, he is cold, and sleepy. He shivers, lies down frequently and stretches full length. In the interval he gets up, huddles in the straw, or with his fellows in the corner of the cage. The respiration becomes more and more feeble and finally disappears. The anatomical changes, fatty degeneration and haemorrhage are usually extreme in this type of case."*

1.2.2.2 Human cardiovascular collapse

Human experience suggests that reactions to ingested allergens differ from reactions following antigen injection or insect sting, with ingested allergens more likely to cause death through severe bronchospasm (hypoxia). Conversely, injected and insect venom allergens are more likely to cause cardiovascular collapse.[67] Extravasation of up to 35% of circulating blood volume within 10 minutes, leading to haemoconcentration and hypovolaemia, has been shown to be a major component of anaphylactic cardiovascular collapse to injected allergen in humans.[45,91] As discussed above, severe but reversible myocardial dysfunction associated with non-specific ST changes in 12 lead ECG has also been reported to injected medications[41,91] and during insect

sting anaphylaxis.[39,41] ECG changes indicating myocardial ischaemia have also been reported with sting anaphylaxis.[39] In one case of iatrogenic anaphylaxis for whom pulmonary artery catheter readings were reported, an increase in pulmonary vascular resistance was noted, although this was some time after reaction onset.[92] Relative bradycardias have also been noted during sting anaphylaxis,[39,40] attributed to hypoxia and anxiety by one group.[40]

Although the presence of mast cells in human cardiac (mainly perivascular) tissue is well recognised,[93-95] cardiac dysfunction during anaphylaxis in humans has been considered to be "exceptionally rare".[96] Even in closely monitored cases, an obvious difficulty in determining the contribution of cardiac dysfunction to anaphylactic shock in humans lies with the problems in dissecting this component from other pathophysiologic effects.[89] In most lethal cases, no detailed clinical observations are made. Therefore, primary cardiac dysfunction cannot be excluded as a significant factor leading to circulatory collapse and death.

Cardiac effects may occur secondary to tissue hypoxia. Thus, tachycardia and diastolic hypotension (leading to reduced coronary perfusion), and hypoxaemia due to upper or lower airway obstruction may combine to cause myocardial ischaemia and infarction. This effect may be either global, or confined to an area already compromised by pre-existing coronary disease. Coronary vasospasm has also been demonstrated in isolated heart models of anaphylaxis, but its apparent restriction to smaller animal models (mainly the guinea pig) has lead to the argument that it is unlikely to be a factor in human reactions.[89] However, coronary vasospasm is difficult, if not impossible to diagnose during human anaphylaxis given the confounding factors outlined above, so it cannot be discounted.

1.2.2.3 Neurological compromise

Severe neurological compromise has been noted to occur during sting anaphylaxis in the absence of cardiovascular compromise, associated with cerebral oedema.[48] Thus, although relatively common neurological features such as dizziness, collapse and incontinence are usually thought to be due to hypotension, mediators (released systemically &/or by effector cells within the brain itself) might have a direct effect on nerve tissue. Supporting this hypothesis, neurological syndromes have been associated with angioedema due to other causes.[97]

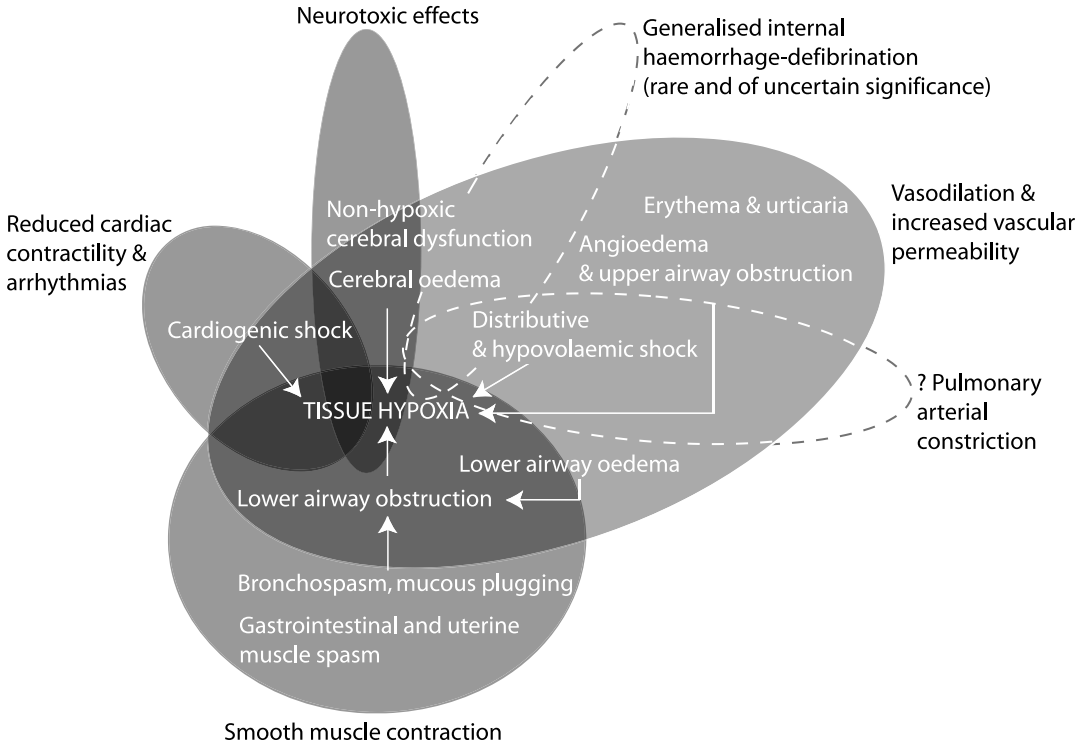


Figure 1.5: Pathophysiology of anaphylaxis.

1.2.2.4 Humans display multiple organ system involvement

Studies of human sting anaphylaxis deaths (see 1.2.4.3 below) are consistent with the concept that in humans, there appear to be multiple potential "shock organs" including the airways, pulmonary circulation, peripheral circulation, heart, blood and brain. Even within a single individual the nature of reactions to the same allergen may fluctuate significantly between predominantly cardiovascular collapse and severe respiratory compromise.[92]

According to the above discussion, pathophysiologic features underlying the four main clinical manifestations of acute human anaphylaxis (Urticaria/angioedema, airway obstruction, shock, and altered consciousness,) are summarised in Figure 1.5. Urticaria, angioedema and upper airway obstruction result from vasodilation ("flare") and increased vascular permeability that cause fluid extravasation and thus tissue oedema ("wheal"). Lower airway obstruction results from a combination of tissue oedema, increased mucous secretion and airway smooth muscle contraction (bronchospasm). A combination of smooth muscle contraction and local oedema also probably underlie uterine contraction pains and the various gastrointestinal manifestations of anaphylaxis.

Anaphylactic shock results from various combinations of blood pooling (distributive shock), fluid extravasation (hypovolaemic shock), cardiac dysfunction and a possible contribution by pulmonary vascular constriction. This may be compounded by hypoxaemia from airway obstruction &/or ventilation-perfusion mismatch, leading to severe tissue hypoxia and worsening myocardial function.

Neurological manifestations, although usually attributed to systemic hypoperfusion and hypoxaemia, may also result from cerebral oedema (vasodilation and extravasation), local alterations in blood flow, and anaphylactic mediator effects at a cellular level. Occasionally, a defibrination syndrome may occur rapidly with massive internal haemorrhage,[98] which may be related to an extreme form of the coagulation changes frequently observed during sting anaphylaxis, but which do not usually result in clinically evident haemorrhage (see 1.2.2.8 below). However, a degree of generalised internal haemorrhage, although not considered a primary cause of death is present in around 27/100 (27%) autopsies in one series,[98] and 19/29 (67%) in another.[99]

1.2.2.5 Delayed large local and "serum sickness" reactions

Both large local reactions and delayed skin reactions appear to be triggered by the same mechanism involved in immediate-type allergic reactions, namely IgE.[100,101] The uncommon "serum sickness"-like syndrome rarely seen following insect stings is also thought to be IgE mediated.[62] There is some evidence that delayed local reactions are associated with heightened cellular responses to antigen.[102] Our knowledge of the actions of mediators released following mast cell activation is compatible with both a prolonged mediator effect and cellular recruitment in the inflammatory response (see 1.2.2.8 below).

1.2.2.6 Triggering mechanisms

In 1968, Coombs and Gell classified anaphylaxis as a "Type I" (anaphylactic or reagin-dependant) hypersensitivity illness, designating cytotoxic (IgG and IgM mediated), Arthus-type (IgM and IgG antibody-antigen complex mediated, including "serum sickness") and delayed (T-lymphocyte cell mediated) reactions as Types II, III and IV respectively.[61] The heat sensitive "reaginic antibodies" implicated in Type I hypersensitivity reactions were formally defined as Immunoglobulin E (IgE) later in the same year.[103] Produced by plasma cells, IgE binds to the surface of effector cells and on subsequent contact with antigen activates them to release preformed and newly generated mediators that cause the clinical syndrome of anaphylaxis. For effector cell activation to occur, two or more antibody binding sites on a single antigenic molecule

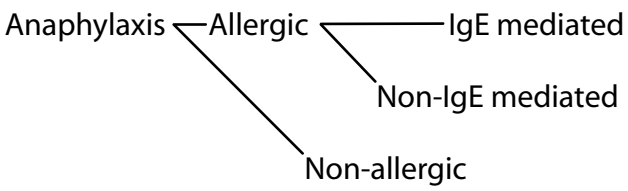


Figure 1.6: Mechanistic classification of anaphylaxis

are required to bring together, or "cross-link" two or more IgE molecules, which are bound to the surface of the cell by the high-affinity receptor for IgE (FcεRI).[104]

A recent position statement by the European Academy of Allergology and Clinical Immunology (EAACI) considers the Coombs & Gell system to encourage too much emphasis on supposedly distinct and mutually exclusive roles of antibodies and immunocompetent cells,[105] which is "not consistent with our present knowledge of the dynamic immune response, as orchestrated by dendritic cells and T helper cells, and mediated by effector cells of several types, antibodies, chemokines, and cytokines."

The EAACI considers that the term "anaphylactoid" (used by some to refer to non-IgE mediated reactions) should not be used, dividing anaphylaxis into "allergic" (immune-mediated) and "non allergic" anaphylaxis, further subdividing allergic anaphylaxis into IgE mediated and non-IgE mediated categories (Figure 1.6).[105] This view is not universally held; an alternate system classifies reactions as either anaphylactic (IgE mediated) or anaphylactoid (non-IgE mediated). Non-IgE mediated mechanisms may be further grouped into those that are due to contact activation of the complement system (eg radio contrast agents), non-specific degranulation of mast cells and basophils (eg opiates, exercise and temperature), immune aggregates (eg intravenous immunoglobulin), cytotoxic (eg transfusion reactions to cellular elements mediated by IgG and IgM) and psychogenic.[81]

Although the majority of people with a history of sting anaphylaxis have demonstrable venom-specific IgE, (sIgE) reactivity, some people suffer a systemic reaction without any recollection of prior exposure and do not have detectable sIgE either by skin testing or RAST.[106] A prospective study of 307 people with a history of systemic reactions found that 99 (32%) had negative intradermal venom skin tests (VST) and 56 (18%) had both a negative VST and negative RAST. Of 14 with negative VST and negative RAST selected for a diagnostic sting challenge on the basis of a "convincing recent reaction history", 2 (14%) had a systemic reaction.[107] Although these findings have led to the suggestion that in some people, reactions may be triggered by non-IgE

mediated mechanisms,[106,108] it is also possible that in these people sIgE is present at a level below the threshold of current diagnostic tests but still capable of triggering systemic reactions.

Further complicating the issue is the observation that 15%-24% of an exposed adult population with no history of systemic reactions will have positive venom skin tests, or "asymptomatic sensitisation" (see 1.2.6 below). With subsequent stings in this group, the risk of a systemic reaction is 17%. The half-life for sensitivity as measured by RAST and VST is around 4 years (i.e. ½ of asymptomatic but VST-positive people will become VST-negative every 4 years).

It is possible that everyone with a sIgE response has the potential to react if stung during a susceptible period when sIgE levels are high. The natural tendency for sensitivity to decline over time, combined with the infrequency of accidental stings and marked variability in the amount of venom injected,[109] may result in the majority of people who develop a sIgE response never experiencing a systemic reaction. A concept relevant here is "allergic breakthrough",[110] which theorizes that development of allergy requires coincidental sensitisation combined with an imbalance in the normal damping mechanism that serves to limit IgE antibody production. However, if levels of sIgE production were "the whole story", then one might expect both the occurrence and severity of anaphylaxis to be linked to serum sIgE levels and/or VST sensitivity, and/or with the interval lapsed between stings, both of which have been demonstrated not to be the case for sting allergy (see 1.2.7).

These findings (asymptomatic sensitisation, severe anaphylaxis in the absence of detectable sIgE, and lack of correlation between serum sIgE and reaction occurrence and severity) suggest that although sIgE may be a critical component of sensitivity, factors other than sIgE are important in determining individual sensitivity to insect stings.

1.2.2.7 Effector cells

Mast cells (named after the term "Mastzellen" or "well-nourished cells" coined by Paul Ehrlich in 1879) were first linked to anaphylaxis in 1941 when the anticoagulation seen in dogs during anaphylactic reactions was demonstrated to be due to heparin, known to be a major component of mast cell granules. Around the same time, histamine was linked to the physiological changes seen in anaphylaxis, and then identified as a component of mast cell granules in 1952.[111] The mast cell, and its circulating counterpart the basophil, are still regarded as having a pivotal role in anaphylaxis.[112] As noted above, activation may occur through either IgE-mediated or non-IgE

mediated mechanisms in response to allergens and other stimuli, leading to the release of preformed and newly generated mediators. Basophils migrating to affected tissues have been implicated in delayed phase reactions.[113]

Other cells thought or suspected to play significant roles in anaphylactic reactions include eosinophils, platelets, monocytes/macrophages and antigen-presenting cells (APC). In animal models of anaphylaxis and human atopic respiratory disease, eosinophils are known to produce a number of pro-inflammatory substances and accumulate in affected tissues.[112] Eosinophils are known to have high-affinity (FcεRI) IgE receptors that enable them to be activated directly by antigen.[114] However, studies looking for evidence of secretory activity during human anaphylaxis have yielded conflicting results.[115-117] Peripheral blood monocytes bear FcεRI and can be directly activated through antigen binding to IgE,[118] although the precise role of this activation remains unclear.

A study of IgE-deficient, FcεRI-deficient, and mast cell-deficient mice has found that anti-IgE-induced anaphylaxis is FcεRI and mast cell dependent, whereas neither mast cells nor platelets appeared important for antigen-induced anaphylaxis.[119] Platelet aggregation occurred, but blocking this did not affect reaction severity. Conversely monocytes/macrophages played a central role in antigen-specific anaphylaxis, possibly involving IgG bound to the surface of these cells via FcγRIII, leading to the suggestion that this mechanism may also be important in human anahylaxis.[119]

The well differentiated or "professional" APCs (dendritic cells and Langerhans' cells) also have FcεRI receptors and so can be sensitised by IgE and release mediators on re-exposure to antigen, attracting more APCs and monocytes to the site of inflammation. APCs appear to be important in the genesis of delayed-type IgE-mediated reactions, but may also play a modulatory role.[120,121]

1.2.2.8 Mediators

Activation of mast cells and basophils via FcεRI leads to the release of a large number of preformed and newly generated mediators with a variety of pathophysiologic actions. The best understood of these mediators and actions are listed in Table 1.2. It should be noted that a number of less well-defined mediators, have not been included. Furthermore, there is significant heterogeneity of mast and basophil cell function between species and within species, such that levels of different mediators my vary depending on the species studied, mode of mast cell activation and cell characteristics.[125,127] However, it is clear that mast cell and basophil responses during anaphylaxis involve large numbers of mediators with a significant amount of redundancy, positive feedback mechanisms

Table 1.2: Mediators of anaphylaxis released by mast cells and basophils, after Williams and others[89,119,122-125]

Mediator	Known actions
Preformed for immediate release	
Histamine	Vasodilation and oedema, bronchoconstriction, mucus secretion, nerve stimulation, reduced myocardial contractility (H1), increased myocardial contractility (H2)
Heparin	Anticoagulant, anti-inflammatory
Tryptase	Amplification of allergic response (positive feedback on effector cell), leukocyte migration and activation, bronchoconstriction, vasodilation and oedema, tissue degradation and cell proliferation ("remodelling")
Chymase	Vasodilation and oedema, mucous secretion, leukocyte activation, tissue degradation
TNF-α	Bronchoconstriction, leukocyte adhesion, leukocyte migration and activation; prominent role in delayed phase reactions
Newly generated over minutes	
Cyclo-oxygenase products; mainly PGD_2	Vasodilation and oedema, bronchoconstriction, mucus secretion, nerve stimulation (→vasodilation, itching, bronchoconstriction)
Lipoxygenase products; LTB_4, LTC_4, LTD_4, LTE_4	Vasodilation and oedema, mucus secretion, bronchoconstriction, leukocyte recruitment
PAF	Platelet activation/microthrombi, leukocyte migration/activation, histamine release (indirectly by neurogenic activation), reduced myocardial contractility
Newly generated over hours	
IL-5, GM-CSF	Leukocyte adhesion, leukocyte migration and activation
IL-4, IL-13	IgE production and up regulation of FcεRI expression

and recruitment of other effector cells and thus further mediator release. This has led to the concept of a "mast cell-leukocyte cytokine cascade" that appears important in the initiation, amplification and perpetuation of allergic responses.[125]

Analysing the roles of these mediators in human disease is difficult because of inter-species variation and the opportunistic nature of anaphylaxis research, given the ethical issues surrounding deliberate provocation of anaphylaxis. Interpretation of the available data is also hampered by the fact that key steps in the allergic reaction occur locally at the site of initial contact and mediators from this component will not be detectable systemically. Furthermore, the relative potencies of various mediators varies enormously; for example, sulphidoleukotrienes are around 1000 times more potent than histamine with regard to airway and vascular effects.[128,129] Therefore, significant clinical effects may occur below the threshold of detection in serum, depending on the analytical technique used. Variable target organ sensitivities may also complicate the analysis of mediator levels.

Table 1.3: Prospective human studies of mediator levels and haematological alterations during anaphylaxis

Study	Population	Number of subjects:			Main findings
		Total	Reacted	Shock	
Smith et al (1980)[39]	Sting challenges	17	10	3	*Plasma histamine;* did not correlate with cutaneous features; in 2 with prolonged shock, peaked early but fell to normal despite ongoing severe anaphylaxis. *Factors V, VIII and fibrinogen;* fell markedly in 2 with prolonged shock reactions. *HMW kininogen, C3 and C4;* fell by ~60% of normal in one subject, minimal change in other subject. *Factor XII and factor B;* no change.
van der Linden et al (1990)[130]	Sting challenges	8	7	4	Falls in albumin and IgG indicating increased vascular permeability. Evidence of complement activation (elevations in C3a/IgG, fall in C1q/IgG and fall in C3/IgG ratios) that was more prominent in those with hypotensive reactions.
van der Linden et al (1992)[131]	Sting challenges	138	39	17	Clinical reaction occurrence and severity correlated with plasma histamine and tryptase elevations at 15 minutes post sting. Change in mean arterial blood pressure across all groups also correlated well with histamine and tryptase levels. Plasma PGD_2 rose in people with severe reactions, but this was not statistically significant.
van der Linden et al (1993)[40]	Sting challenges	138	39	17	Increases in endogenous adrenaline, noradrenaline and angiotensin II levels in subjects with hypotension. Dopamine and angiotensin I levels did not change in any subjects
Van der Linden et al (1993)[132]	Sting challenges	16	12	4	Decreased kallikrein and HMW kininogen levels (consumption). Increased C1-inhibitor complexes (kallikrein- and factor XIIa-C1-inhibitor) closely associated with angioedema and also shock, and correlated significantly with plasma levels of tryptase and histamine.
Van der Linden et al (1993)[133]	Sting challenges	55	20	7	Von Willebrand factor levels increased in those with hypotension, accompanied by increased levels of tissue plasminogen activator and plasminogen-antiplasmin complex levels. D-Dimers and platelets stable, indicating minimal activation of clotting system.
Lin et al (2000)[134]	Food & drug reactions	97	97	2	Elevated plasma histamine and total tryptase above normal range in 42 and 26 respectively. Detectable β-tryptase in 23. Histamine levels correlated with heart rate and extent of urticaria.

Several prospective studies of serum mediator levels during human anaphylaxis have been published, mostly in the setting of deliberate insect sting challenges.[39,40,130-134] The main findings of these studies are summarised in Table 1.3. Van der Linden's group found clear evidence of mast cell degranulation –raised tryptase and histamine levels above baseline– in nearly all severe sting challenge reactions, with only one of 17 severe reactions having no detectable rises in histamine and tryptase levels.[131] Other investigators have demonstrated, on the basis of changes in circulating leukocyte histamine content, that histamine is released from basophils as well as mast cells during sting anaphylaxis.[135] Human sting anaphylaxis is associated with activation of the contact system (kallikrein activation, leading to cleavage of high molecular weight kininogen). This in turn leads to elevated bradykinin levels, for which the more stable C1-inhibtor complex is used as a marker,[132] activation of the fibrinolytic system,[133] and activation of the complement system.[39,130] Activation of the clotting system may also occur, as evidenced by reductions in factors VIII, V and fibrinogen in one study,[39] however van der Linden's group suggested this effect to be relatively minor on the basis of their results showing little change in d-dimer levels and platelet counts.

The mechanisms of these changes are uncertain. Suggestions have included direct activation of kallikrein and factor XII by mast cell mediators (perhaps including heparin and chondroitin sulphate E), release of a kallikrein-like substance by basophils,[132] and release of endothelial tissue plasminogen activator and von Willebrand's factor in response to hypoxaemia, acidosis, elevated catecholamine levels, histamine and other mediators released by mast cells and basophils.[133] However, whatever the mechanisms involved, it appears that the manifestations of sting anaphylaxis in humans result from the actions of a number of mediators, amplified by various factor cascades and positive feedback mechanisms, and with significant heterogeneity of mediator release patterns between individuals.

A number of studies have demonstrated that histamine infused into both healthy volunteers and animals can alone cause all of the clinical findings observed during anaphylaxis; interestingly histamine may have beneficial effects on contractile function via H2 receptors.[89] However, severe cardiovascular compromise in humans has been observed to last far beyond the peak in serum histamine levels,[39] and antihistamines have been felt by clinicians to provide little benefit in a large series of cases of severe anaphylactic shock under anaesthesia.[45] Furthermore, in both guinea pig and canine models antihistamines are able to prevent the changes induced by histamine infusion but do not prevent the cardiovascular changes seen during anaphylaxis,[86,136] and in a monkey model death can occur with insignificant amounts of histamine release.[87] In guinea pigs, histamine appears to mediate immediate cardiac changes (ischaemia and

pump failure) but antihistamines provide some protection for the first few minutes only, whereas platelet activating factor (PAF) is the major mediator after this. Treatment with a PAF-antagonist in combination with antihistamines significantly ameliorated contractile failure.[136] Recent murine studies suggest that PAF release by macrophages in response to surface IgG receptor (FcγRIII) cross-linking is an important anaphylactic pathway, and that this may be important in some human reactions.[119]

Human heart mast cells have been isolated and on FcεRI cross-linking release tryptase, chymase, histamine, prostaglandin D2 (PGD2) and leukotriene C4 (LTC4), [95] in significantly different proportions (more LTC4 than PGD2) when compared to cultured skin and lung mast cells, indicating significant heterogeneity in function.[93] In addition to being synthesized by mast cells and basophils, PAF is synthesized by human endothelial cells in response to histamine and persists for 45 minutes after the stimulus has been removed.[126]

1.2.3 Management

In addition to standard resuscitative measure (supine position, airway management, ventilatory support and external cardiac massage as required), effective management of anaphylaxis requires antagonism of the effects of anaphylactic mediators, reversal of intravascular volume depletion with aggressive fluid resuscitation,[45] and occasionally circulatory support with mechanical assist devices such as the intra aortic balloon pump.[41]

The large number of anaphylactic mediators suggests that antagonism of one or even several individual mediators is unlikely to be effective. An example of this is the demonstration that antihistamines reverse the effects of histamine infusion that mimics anaphylaxis, but are ineffective in treating anaphylaxis mediated by mast cell degranulation.[86,136]

1.2.3.1 Adrenaline

Adrenaline was noted to be useful in the treatment of anaphylactic shock as early as 1925,[137] and was considered the "natural antagonist of histamine",[138] at the time thought to be the principal mediator of anaphylaxis. On the basis of expert opinion, the importance of adrenaline in emergency treatment continues to be emphasized in contemporary resuscitation guidelines.[139-141] Adrenaline is a "physiological" antagonist of the changes seen during anaphylaxis (rather than a mediator receptor antagonist), acting by reversing peripheral vasodilation and oedema (α-receptor stimulation), cardiac stimulation (β_1-receptor stimulation), and bronchodilation (β_2-receptor

stimulation). Studies on isolated rat peritoneal mast cells also suggest that adrenaline may act to reduce further mediator release,[142] although these findings have not been confirmed for human mast cells.

There have been no prospective comparative evaluations of adrenaline in the management of human anaphylaxis. One large analysis of anaphylactic reactions under anaesthesia concluded that adrenaline combined with fluid resuscitation is an effective treatment approach, and lethal outcomes appeared to be restricted to cases where adrenaline was not used until late after the onset of symptoms.[45] Other studies have also associated failure to use adrenaline early as a major factor contributing to lethal outcomes.[54,67] However, sting challenge studies suggest that many severe reactions eventually improve with fluids and antihistamines alone.[40]

Recurrent debate on the optimal route of administration has lead to editorial commentaries that emphasise the need to adapt to prevailing clinical conditions (clinical urgency, degree of circulatory compromise which may limit absorption, the availability of vascular access, and the level of care available) which may predicate one route or method of administration over another.[96,143] Where the circulation is intact (and in the absence of anaphylaxis), absorption of a 0.01 mg/kg dose of adrenaline in children and 0.3 mg in adults is maximal after intramuscular administration but is unreliable and delayed after subcutaneous administration.[144,145] Interestingly, intramuscular administration of 0.3 mg in 0.3 ml (1:1000 adrenaline) into the arm appears to be no better than subcutaneous administration in young adults, whereas injection into the large muscle of the thigh results in rapid and reliable absorption.[145]

Based on available data as well as theoretical considerations, the prompt administration of an intramuscular dose of adrenaline prior to establishing intravenous access is generally accepted as an appropriate first step in the management of anaphylaxis.[139-141] However, clinical observations of severe anaphylaxis in humans,[39] as well as canine experiments,[146] suggest that a single dose of adrenaline produces only transient improvements in cardiovascular parameters during anaphylaxis. Therefore, multiple intramuscular doses may be required and once intravenous access is established, consideration must be given to establishing a continuous infusion of adrenaline in severe cases.

Although it has been reported that large doses (20-30 puffs) of an adrenaline inhaler (Medihaler Epi) in adults results in a more reliable and rapid systemic absorption of adrenaline than subcutaneous injection,[147] inhalations appear to be unreliable in children.[148] Their use has also been cautioned against in adults due to the need for large

numbers of inhalations and short duration of effect.[149] Recently, the Medihaler Epi has been withdrawn due to stability concerns and it appears that there will be no attempts to return this formulation to the market.[150]

1.2.3.2 Other sympathomimetics

Where anaphylaxis has been resistant to adrenaline and aggressive fluid resuscitation, the successful use of noradrenaline, amrinone and glucagon have been reported.[45,151,152] Noradrenaline may produce are more intense activation of adreno-receptors (particularly peripheral alpha receptors) to reverse the vascular effects of anaphylaxis. Glucagon and amrinone bypass beta-receptors to increase intracellular levels of cyclic AMP, thus potentially reversing bronchospasm and increasing cardiac output. This later effect has also been postulated to be of potential use where anaphylaxis occurs in the setting of beta-blockade.[143]

1.2.3.3 Steroids, antihistamines and other antagonists

Although both antihistamines and steroids are recommended as second-line management after adrenaline and fluid resuscitation, little data exists to support this approach. The use of steroids is based on theoretical considerations and their proven role in the management of asthma, where suppression of tumour necrosis factor (TNF) secretion by inflammatory cells may be a key mechanism of action.[125] As a delayed (>1 hour) effect is to be anticipated from steroids, current recommendations are that they be reserved for severe anaphylaxis where the risk of relapse (delayed phase reaction) is probably high.[140,141]

Human and animal histamine H_1 receptors appear to mediate vasodilation, tachycardia and decreased left ventricular function, whereas H_2 receptors mediate increased LV contractility. Thus from a cardiac perspective the use of H_2 blockers to treat anaphylaxis is controversial.[89] However, in mild allergy, a combination of H_1 and H_2 blockers appears to be superior to H_1 blockers alone for the treatment of cutaneous manifestations; it should be noted that the results of this study appear to be confounded by higher adrenaline usage rates in the combination H_1 plus H_2 blocker group.[153] The plethora of effector systems (cytokine-cellular cascade), the profound cardiovascular depression that extends well beyond the early peak in serum histamine,[39] and animal studies,[154,155] indicate that histamine receptor blockers are of little use in the management of anaphylactic shock. Nevertheless, consensus guidelines continue to recommend either H_1 blockers alone,[139,140] or in combination with H_2 blockers.[141]

Although animal studies suggest combination histamine receptor blockade, leukotriene and PAF antagonism could be useful in the management of anaphylactic shock,[136] no human studies have been reported.

1.2.4 Mortality

1.2.4.1 Incidence

Estimates of the incidence of insect sting anaphylaxis deaths appear to vary according to population exposure (determined by local climate and insect species). Medical awareness of anaphylaxis as a potential cause of death may also influence mortality figures, so published mortality rates are likely to be under-estimates. At post mortem there are no features to indicate an allergic death in around half of all cases dying from anaphylaxis, so a careful review of clinical information is essential and serological investigations may be required.[156] In the UK, where a specific register of anaphylaxis deaths was set up in 1992, of 164 referrals to the register where death was clearly due to anaphylaxis, 35 death certificates (21%) had not listed anaphylaxis as a cause or contributing factor because of absent diagnostic post mortem findings. Investigations with potential to clarify diagnosis, such as sIgE serum analysis and serum tryptase levels were also rarely performed.[67] Similarly in Australia, a number of sting anaphylaxis deaths have not been diagnosed as such in death certificates.[66]

United States of America (USA)

For the whole of the USA, incidences of 0.14 and 0.18 per million of population per year have been reported in 1963 and 1997 respectively, with the vast majority due to bees, vespid wasps, and paper wasps.[157,158] Most deaths occurred in the warmer southern states where in 1963 the incidence was estimated at 0.39 per million per year. No statistics are available for the incidence of death due to IFA sting in the USA since the widespread establishment of this introduced pest, largely because of the absence of a specific mortality code to differentiate this cause. Eighty-three deaths, claimed to be caused by IFA but accompanied by minimal clinical data, have been reported.[159]

Europe

Mortality rates reported from Europe range from 0.2 to 0.45 deaths per million per annum.[11,65,160] In Sweden, a mortality rate of 0.2 appears primarily due to wasps (25 of 26 deaths),[160] whereas in Denmark where a mortality rate of 0.26 has been reported, honeybees and wasps were reported responsible for 9/26 (35%) and 15/26 (58%) respectively (in the remaining 2 cases, the cause was uncertain). In Switzerland, with a reported sting anaphylaxis mortality rate of 0.45, honeybees are responsible for

the majority (approx 67%) of insect sting allergy where the causative insect can be identified.[11]

Australia and Tasmania

No overall mortality rate for insect sting anaphylaxis in Australia has been published, however death rates to honeybee sting have been reported at 0.086 per million per annum,[66] wasps at 0.02,[161] and ants at 0.02.[162] Not surprisingly, regional differences are substantial and reflect local climate, insect populations, agricultural and recreational activities.[66] Honeybee sting mortality is especially common in South Australia (mortality rate 0.26 per million per annum).[66] Despite the establishment of *Vespula germanica* in Tasmania and Victoria, wasp sting mortality in Australia seems to be exclusively due to paper wasps (Polistes and Ropalidia) and confined to northern New South Wales and Queensland.[161] *Myrmecia* ant sting mortality appears to be concentrated on the island of Tasmania (5 deaths in 20 years, out of a national total of 6).[162]

It is likely that deaths due to *Myrmecia* spp. have been significantly under-reported. A very recent discussion of insect sting allergy in the Australian forensic pathology literature completely ignored the possibility of ant sting allergy as a cause of death.[163] Furthermore, sudden unexplained deaths in rural areas where *Myrmecia* are common may not always be well investigated, compared to deaths occurring in urban areas serviced by specialist facilities (where bees and wasps are more likely to be encountered than *Myrmecia* spp.).

1.2.4.2 Factors predisposing to death

Studies that systematically examine insect sting deaths are summarised in Table 1.4. The majority of deaths occur in males over 30 years of age. This differs from snakebite deaths, which are equally distributed across age groups.[157] A precipitous increase in deaths over the 30-year mark and the predominance of males may be due to a number of factors. Allergy prevalence is known to increase with age,[168] and males may also be more heavily exposed to stings by virtue of employment and recreational activities. Age also appears to be a risk factor for reaction severity, with people experiencing hypotensive sting challenge reactions being older than those who do not (mean ages of 48 years and 28 years respectively for honeybee allergy).[52]

Prior known sting allergy has been recorded for only 14% of deaths overall. One larger study noted that reference to prior known allergies is frequently missing from coronial records.[98] However, selection bias may have an opposite effect, as known allergy is

Table 1.4: Insect sting mortality; age, prior known allergies, and comorbidities

Study	Place; insects	Number of sting deaths	Prior known systemic allergy	Age (years)				Sex	Comorbidities	
				<10	≥10 to <30	≥30 to <50	≥50	M:F	Asthma	CVS
Parrish[157]	USA; B, W, A	229	NR	13 (6%)	13 (6%)	90 (39%)	113 (49%)	2.8:1*	NR	NR
Hunt[164]	Virginia; B, W	18	8 (44%)	3 (17%)	2 (11%)	6 (33%)†	7 (39%)	2:1	2	4
Lecomte[165‡]	Europe & USA; B, W	143	28 (20%)	6 (4%)	7 (5%)	70 (49%)	60 (42%)	2.5:1	2	9
Somerville[166]	Eng/Wales; B, W	61	NR	2 (3%)	2 (3%)	21 (34%)	36 (59%)	1.2:1	NR	NR
Mosbech[65]	Denmark; B, W	26	5 (19%)§	1 (4%)	1 (4%)	10 (38%)	14 (54%)	2.7:1	1	9‖
Harvey[66]	Australia; B	25	NR	5 (20%)	3 (12%)	10 (40%)	7 (28%)	4:1	NR	NR
Nall[167] and Barnard[98¶]	USA; B, W	677	61 (9%)	2%	NR	NR	48%	4:1	NR	NR
Sasvary[99]	Switzerland; B, W	29	10 (34%)	0	0	All aged >36 years		3:1	13	24
Pumphrey[67]	UK; B, W	23	4 (18%)	NR	NR	NR	NR	1:1	NR	NR
McGain[161,162]	Australia; W & A	13	9 (69%)	0	0	All were males aged >30			1	3
	Amalgamate (%)		126/929 (14%)	30/502 (6%)	28/502 (6%)	207/502 (41%)	237/502 (47%)	3.5:1	19/229 (8%)	49/229 (21%)

NR; Not reported. B=bee, W=wasp, A = ant. Mortality studies reporting inadequate clinical data have been excluded.[158,159] *M:F ratio quoted is for all venomous animal deaths (specific figure not quoted for Hymenoptera). †Some discrepancies in tables- 1 case aged >30 (precise age not stated) placed in this group but may belong in >50 group. ‡ Consists a detailed review of all prior case reports (237 deaths) including original data from Barnard, Hunt and Parrish studies which have been subtracted to avoid double-counting, along with cases reporting inadequate clinical data, leaving 143 for analysis. § Severe (grade III/IV) in 3; “unwell” in 1; “severe urticaria” in another. ‖Only 5 were considered to have severe disease likely to affect outcome. ¶ Nall’s study includes data reported previously reported in greater detail by Barnard (1973).

more likely to flag a potential anaphylactic death. Therefore, the figure of 14% may be accurate, and the first reaction may present the greatest risk of death to allergic individuals. Catastrophic (invariably lethal) reactivity might be largely confined to individuals who do not survive to receive a repeat sting. Furthermore, sting avoidance, emergency treatment measures and more recently venom immunotherapy may effectively reduce the risk of death in those who survive their first reaction.

Although comorbidities are often cited as a factor in sting allergy deaths, respiratory and cardiovascular comorbidities respectively have been identified in only 8% and 21% of deaths overall, and may not be severe. Comorbidity rates in age- and gender-matched control groups are not presented in these studies, raising the possibility that co-morbidities are only secondarily associated with mortality, with age being the major predictor of reaction severity.

Mechanistic studies and case reports have suggested angiotensin converting enzyme (ACE) inhibitors and β-blockers may play a role in the occurrence of severe anaphylaxis. ACE inhibitors can trigger severe anaphylaxis in patients undergoing venom immunotherapy,[169] whilst case reports of anaphylaxis and systemic reactions occurring during immunotherapy suggest that β-blockers may both impair the endogenous adrenergic stress response and counteract and imbalance the effect of both endogenous and exogenously administered catecholamines at the receptor level.[151,170-174] Nevertheless, mortality studies have not established any link between death and therapy with these agents.

1.2.4.3 Mode of death

The majority of deaths (57-94%) occur within 1 hour of the sting;[98,157,164,165,167] and the median time from sting to onset of cardiac arrest in fatal cases is 15 minutes.[67] However, significant delays of can occur in the onset of symptoms and cardiac arrest may be delayed for several hours.[67,99]

Early studies of large numbers of deaths denoted primary cause of death based on post mortem findings. Using this approach, the primary pathology is “respiratory” (upper and lower airway oedema/obstruction), “anaphylaxis” (clinical shock picture +/- less severe respiratory pathology), “vascular” (coronary occlusion, generalised haemorrhage &/or organ engorgement and embolic disease) and “neurological” (oedema, haemorrhage, infarction, necrosis and degeneration) in 54%, 34%, 8% and 4% of deaths respectively.[167,175] Some neurological cases might have been secondary to cerebral hypoxia. However one case had marked cerebral oedema and died within minutes (making hypoxia an unlikely cause of the cerebral oedema, although shock

may have been present), and in only one of the remaining 6 cases who died after one or more days was the cerebral pathology clinically diagnosed as being due to cerebral hypoxia.

Smaller mortality studies providing greater clinical detail suggest that death is often due to a mixed respiratory/circulatory compromise, and that a number of people with marked airway oedema also succumb suddenly, presumably due to cardiovascular collapse.[65,161,162] Between 9 and 15% of sting deaths have been reported as most likely to be non-allergic upper airway obstruction, almost exclusively due to wasps.[65,67,165,166] Wasps are attracted to fruit and sweet drinks, and thus are more likely to enter the mouth accidentally. However, in hot conditions (>35ºC) frequently encountered in Australia, bees forage more for water, and thus may also cause death in this manner.[66] Pumphrey's recent careful review of UK anaphylaxis deaths found that of 32 sting deaths, 15 (47%) were due to shock alone, 13 (41%) were associated with marked respiratory compromise (including 3 who possibly died mainly by asphyxiation from local swelling following stings to the upper airway) and 4 (13%) were noted to have a mixed picture. This pattern contrasted significantly with food allergy deaths where all presented with significant respiratory features; none presented with shock alone and only 5/37 (16%) exhibited a mixed picture.[67] The same study noted the median age for venom allergy deaths to be 54, whereas those for nut and food allergy where 22 and 24 years respectively.

1.2.5 Pathogenesis

Insect sting allergy should be clearly distinguished from atopy. Atopy is a tendency (often inherited) to develop sIgE on exposure to environmental aeroallergens, and to suffer typical allergic respiratory and skin manifestations (allergic rhinitis, bronchial hyper-responsiveness and eczema). It is generally accepted that atopy begins during early childhood with a "TH_2" type cytokine response by T helper (TH) cells in response allergen exposure, probably facilitated by interactions with antigen-presenting cells (APC). This results in the generation of sIgE. Ongoing stimulation appears to cause an "atopic march" with consolidated TH_2 type responses and thus the expression of atopy, that is IgE-mediated reactions to multiple allergens.[176,177] The "hygiene hypothesis" proposes that early in life the immune system is "primed" towards TH_2 responses in atopic individuals due to reduced exposure to certain pathogens. This theory has been challenged as being somewhat simplistic, and an alternate theory proposed that the expression of atopy is determined by continual interaction between microbial, non-infective and lifestyle factors over an individual lifespan.[178]

Although insect sting allergy is IgE mediated, and despite observations that atopic individuals have higher rates of RAST and skin test reactivity to insect venoms,[179] there is no evidence supporting an increased risk of clinical sting allergy in atopic individuals.[105] One study has indicated that atopic individuals, if sensitised to insect venoms, are more likely to experience severe reactions than non-atopics with insect venom allergy,[180] but this finding has not been reproduced by others.[52] Insect venom allergy tends to spontaneously improve if it first arises in childhood with more severe allergy surfacing in later adulthood (see 1.2.7), whereas atopic disease arises early in life and becomes more severe during childhood and early adulthood. Some differences between atopy and venom allergy may relate to the continual topical exposure of mucous membranes to aeroallergens in atopic individuals from very early in life, versus infrequent and irregular intradermal exposure to venom allergens that does not generally begin until the toddler and pre-school stages of development.

As with atopy, the development of an allergic IgE response to venom is thought to require a T helper cell response deviated towards the production of "TH_2" cytokines. The reasons that individuals respond in this way are obscure but risk appears to increase with age in exposed populations,[168] and the risk of sensitisation increases substantially with repeated exposure,[179] suggesting that with each repeated exposure the cumulative risk of encountering a TH_2 response to antigen increases. Polarisation towards a TH_2 response is probably driven by antigen-presenting cells, although the mechanisms by which this occurs remain unclear.[121] Why some peptides and proteins are common allergens but others are not is also unknown; a novel hypothesis, backed by some preliminary data, is that potential allergens are characterized by an ability to cause histamine release by lymphocytes independently of IgE, along with IL-4 release.[181] A subsequent "TH_2" type response may be promoted by both IL4 (see above) and histamine itself.[182,183]

Some evidence suggests that compared to the general population, venom allergic individuals have kinin system hyperactivity (increased kallikrein levels),[184] and reduced renin-angiotensin system activity.[185] It is also intriguing to note that activity of the renin-aldosterone system falls with advancing age.[186] Kinin system activation occurs during sting anaphylaxis (see 1.2.2.8 above), ACE inhibitor therapy appears to be able to precipitate severe anaphylaxis during immunotherapy,[169] and advancing age increases the risk of both hypotensive reactions and death (see 1.2.4.2 above). Taken together, these observations suggest the possibility that endogenous activities of the opposing kinin and renin-angiotensin systems, which decrease and maintain/elevate blood pressure respectively, may be important in the expression of insect sting anaphylaxis in the presence of IgE sensitisation.

1.2.6 Prevalence & causative insects

Studies have consistently demonstrated sensitisation prevalences (defined by serum IgE or skin test reactivity specific to local insect venoms) in the general population of 15-24%, few of whom have ever experienced a systemic reaction.[187-191] In the only prospective study to date, of 65 adults with asymptomatic sensitisation (sIgE present, but no history of allergic reactions) who were later stung, only 11 (17%) had a systemic reaction. The majority never experience a systemic reaction and sIgE levels fall with 1 in 2 becoming non-reactive on RAST every 4 years.[192]

Estimates of prevalence for clinical systemic reactivity (history of a systemic allergic reaction to insect sting at any time in the past, with or without evidence of sIgE reactivity) range from 0.8-3.9%.[187,188,190,193-196] Causative insects and sting exposure rates vary, and some of these results come from selected populations at higher risk than the general population, for example scouts with high outdoor activity levels,[193-195] adults,[187,188,196] and people from rural areas.[190] Interpretation is also influenced by questionnaire design and whether a physician has confirmed the history of systemic allergy.[179] The prevalence of venom allergy appears to be higher in adults than children,[168] and in some studies is more prevalent in males than females, possibly due to male dominance in outdoor activities at the time these studies were performed, a proposition supported by the equal prevalences seen in male and female scouts.

Ant sting allergy is an emerging problem due to the imported fire ant *Solenopsis invicta* in the USA, *Pachycondyla* spp. in Asia and the Middle East and *Myrmecia* spp. in Australia. Two studies have looked specifically at the incidence of clinical ant venom allergy, although no estimate of imported fire ant allergy prevalence has been reported from the USA. In *Pachycondyla chinensis* infested areas of Korea, around 2.1% of the adult population give a history of systemic reactions and positive skin prick tests,[16] whereas in rural areas of Victoria, 2.9% of adults report a previous systemic allergic reaction to *Myrmecia* spp. stings.[28]

Myrmecia pilosula appears to be associated with the vast majority (approximately 90%) of ant venom allergy in Australia, on the basis of both clinical data,[1,2,28] and analysis of allergic sera.[27] Ant sting allergy appears to be uncommon outside of the southeastern states.[1,4] Although honeybee sting allergy appears to be the predominant allergy in Western Australia, the single study from that state appears not to have investigated the possibility of other insects being involved,[190] even though *Myrmecia* spp, including *M pilosula*, are known to be found in that region.[26]

1.2.7 Natural history & predictors of reaction severity

Although people tend to have reactions that are similar to previous reactions,[38] reaction severity tends to fluctuate over time.[34,37] A prospective study of 242 children aged 2-16 years with a history of mild systemic reactions (approximately ½ each to honeybee and *Vespula* spp. venoms) has demonstrated that this group has only a 9% (18/86) risk of systemic reaction on subsequent stings; 16 of these reactions were less severe, 2 were of similar severity, and none were more severe.[197] Fluctuations in reaction severity may be due at least in part to variability in the amount of venom delivered. In adults, two large honeybee and wasp sting challenge studies have confirmed that the overall risk for a reaction on subsequent stings for adults with a history of a systemic allergic reaction is around 27% (215/803).[52,198] Both studies are remarkably consistent and show that the risk is higher for honeybees (44-52%) than for wasps (17-25%). Observed differences between honeybee and wasp sting allergy may be due to the amount of venom injected. Bees deliver 59 +/- 7 ug venom with each sting, whereas for *Vespula* spp. the amount delivered is 1.7-3.1 ug.[199] Other than insect species, only prior reaction severity and age were predictive of sting challenge reaction severity. The highest reaction risk was in those with a history of severe (hypotensive) reactions. The mean age of hypotensive reactors was 48 versus 28 for others. Gender, atopy, sIgE level, skin test sensitivity, time interval elapsed between last sting, and number of previous stings were not predictive of either occurrence or severity of a sting challenge reaction. The finding that time elapsed from previous stings is not predictive fits with earlier retrospective observations that patients may react after an interval as long as 55 years,[34] and recent findings that sIgE reactivity can persist for many years,[192] with mast cells maintaining IgE memory long after serum IgE disappears.[200]

Amalgamating data from both studies, in 692/803 (86%) of cases, the reaction to sting challenge was less severe than previous worst reactions. Van der Linden's group found that in none of 324 cases did sting challenge reaction severity exceed that recorded for previous stings.[52] Conversely, Blaauw's group identified 23/479 patients (5%) –mainly those with honeybee sting allergy– in whom sting challenge severity was worse than the previous worst stings.[198] Reasons for this discrepancy between two large and similarly conducted studies using exactly the same grading system are not apparent.

Published data does not permit an evaluation of whether increased age and prior reaction severity independently influence subsequent reaction risks. It is possible that age at the time of first reaction determines the risk of a severe reaction, with this being the most important predictor of subsequent reaction risk. In addition, there may be an interaction over time, with increasing age causing reactions to become more severe.

A study of patients requiring increased maintenance doses during honeybee and wasp venom immunotherapy to provide protection from sting challenges noted a disproportionate number of such patients to have elevated baseline tryptase levels.[201] The same group found elevated baseline mast cell tryptase and clinical evidence of mastocytosis to be associated with a history of severe honeybee and wasp sting reactions.[202] It may be that both clinically apparent and sub-clinical degrees of mastocytosis may be associated with mast cell instability and a tendency towards more severe reactions.

No retrospective or prospective studies have examined the natural history of clinical allergy or predictors or reaction severity in people with ant sting allergy.

1.3 Anaphylaxis classification and grading systems

Anaphylaxis grading systems are examined here in detail, as an understanding of these is required to guide experimental designs where reaction severity is a major outcome.

In a study of emergency department presentations, Brown et al define reactions limited to the skin (including angioedema) as “acute allergic reactions”, reserving the term “anaphylaxis” for reactions with additional gastrointestinal, respiratory, cardiovascular or neurological features; their three-level grading system is presented in Table 1.5.[203] This system is notable for an approach that focuses on detailed criteria for severe anaphylaxis, with the remaining cases easily classified as either an “acute allergic reaction” or “mild-moderate anaphylaxis”. However, the detailed physiological criteria may be difficult to apply in retrospect when analysing cases that occur outside a closely monitored environment. Also, the “severe category” gives the same weight to well-defined physiological criteria (blood pressure, respiratory rates, level of consciousness) as to subjective criteria such as dizziness and light-headedness.

A survey of published clinical studies using grading systems to describe sting reactions reveals a number of approaches with varying complexity. Arguably the simplest, but rarely used system defines “slight” by itch and urticaria only, “severe” by dyspnoea, upper airway obstruction or shock and “moderate” as those falling “in between” the other two groups.[37] Reisman also uses a simple, yet better defined three-grade classification (Table 1.6).[38] Another well-known system, presented by Harry Mueller in 1959 (Table 1.7),[35,36] is frequently quoted. This is notable for providing both a well-defined grading system and a reasonably comprehensive list of common reaction features. In 1990, Ulrich Müller published a substantial modification that more clearly

Table 1.5: Grading system for general emergency department presentations with acute allergic reactions according to Brown et al[203]

Grade	Features
Acute allergic reaction	Evidence of generalized mediator release restricted to cutaneous findings alone (generalized rash, pruritus, rhinitis/conjunctivitis, urticaria, local edema, and angioedema) without any other systemic symptoms or signs.
Mild-moderate anaphylaxis	*Any of the above plus* additional respiratory, cardiovascular, gastrointestinal, or neurological features (including; dyspnoea, wheeze, hoarseness, nausea, vomiting).
Severe anaphylaxis	*Any of the above plus* potentially life-threatening symptoms or signs, namely; A history of loss of consciousness, syncope, dizziness or light-headedness at any time; systolic blood pressure on arrival in the ED or within 30 minutes of arrival of <90 mmHg; a Glasgow Coma Scale score on arrival or within 30 minutes of arrival of <15, related to cardiovascular system collapse and/or neurological dysfunction from hypoperfusion, hypoxia, and the direct effect of mediators, or; Shortness of breath, wheeze, hoarseness or bronchospasm plus any 1 or more of stridor, cyanosis, laryngeal edema or a respiratory rate ≥25/min on ED arrival or within 30 minutes from respiratory system dysfunction.

differentiated gastro-intestinal, respiratory and cardiovascular reaction features in order of increasing severity (Table 1.8).[11]

Large sting challenge studies published since 1994,[52,198,204] although quoting Harry Mueller's system appear to have in fact used major modifications closer to those outlined by Ulrich Müller. Of note, these sting challenge studies further simplified the application of grades by removing various subjective symptoms from the classification, as well as the "two or more" rules (Table 1.9). Angina pectoris and arrhythmias were also added to grade IV. Clearly this approach is suited to close observation of a reaction in a controlled environment. However, the "two or more" rules used by both Mueller and Müller may be a useful strategy when retrospectively attempting to judge the more subjective features that are likely to be of little significance when occurring in isolation. Angina pectoris and arrhythmias, although undoubtedly serious, could be argued to reflect complications related to underlying comorbidities rather than reaction severity *per se*.

Some investigators have ignored the gastrointestinal manifestations of sting reactions.[205,206] The system used by Golden's group,[206] similar to that previously described by Settipane and colleagues,[205] simply grades systemic reactions as "minimal", "generalised urticaria &/or angioedema only", "respiratory" or

Table 1.6: Grading system according to Reisman[38]

Grade	Features
I (mild)	Dermal (urticaria, angioedema)
II (moderate)	Dermal plus “other non-life-threatening features such as mild asthma or dyspnoea”
III (severe)	Hypotension, shock, loss of consciousness, upper airway oedema, severe respiratory distress

Table 1.7: Grading system for insect sting anaphylaxis according to H. L. Mueller[35,36]

Grade		Features
1	Slight general reaction	Generalised urticaria, itching, malaise and anxiety
2	General reaction	Any of above plus 2 or more of the following: generalised oedema; constriction in chest; wheezing; abdominal pain; nausea & vomiting; and dizziness.
3	Severe general reaction	Any of the above plus 2 or more of the following: dyspnoea; dysphagia; hoarseness or thickened speech; confusion; and feeling of impending doom.
4	Shock reaction	Any of the above plus 2 or more of the following: cyanosis; fall in blood pressure; collapse; incontinence; and unconsciousness.

Table 1.8: Grading system for insect sting anaphylaxis according to U. R. Müller[11]

Grade	Features
I	Generalised urticaria, itching, malaise and anxiety
II	Any of the above plus angioedema or at least two of; constriction in chest, nausea, vomiting, diarrhoea, abdominal pain or dizziness.
III	Any of the above plus dyspnoea, wheezing or stridor, or at least two of; dysphagia, dysarthria, hoarseness, weakness, confusion, feeling of impending disaster.
IV	Any of the above plus two or more of; fall in blood pressure, collapse, loss of consciousness, incontinence, cyanosis.

Table 1.9: Grading system for anaphylaxis used in deliberate sting challenge studies since 1994[52,198,204]

Grade	Features
I	Skin symptoms (generalized urticaria, itching, erythema) or anxiety
II	Gastrointestinal symptoms (stomach pain, nausea, vomiting) or angioedema
III	Respiratory symptoms (dyspnoea, difficulty in swallowing, hoarseness, stridor)
IV	Cardiovascular symptoms (hypotension that "requires immediate intervention", cyanosis, collapse, arrhythmias, or angina pectoris) *

"cardiovascular" (Table 1.10). It is notable that Golden's classification uses cough and dizziness as markers for bronchospasm and hypotension respectively.

Sturm and colleagues[207] applied a system initially used to describe reactions to intravenous medications by Ring and Messmer.[208] This allocates varying skin gastrointestinal, respiratory and cardiovascular reaction features across grades (Table 1.11). The useful feature of this approach is the recognition that a reaction can involve all organ systems yet still range in severity. However, hypotension features in 3 out of the 4 categories. This system is thus oriented towards the very severe end of the spectrum.

Lockey and co-workers describe a system that gives a simple 3-level grade and provides a "systemic reaction index" (SRI) valued between 0 and 1. The SRI for a reaction is the sum of values corresponding to the various reaction characteristics noted to occur (Table 1.12).[209] The design was such that the sum of all values for mild features would not be equivalent to the value for any single moderate feature and in turn, the presence of all moderate features, either alone or together with all mild features, would not give a value equivalent to any single severe reaction feature. As with most other systems, the assumption is made that hypotension is a more severe feature than bronchospasm.

Each of the systems discussed had advantages and disadvantages. An "ideal" system would; (i) be well-defined, reproducible and easy to apply both retrospectively with limited data as well as in a controlled "data-rich" clinical environment; (ii) account for the potential importance but subjectivity of historical reaction features such as dizziness, weakness, and feeling of impending doom; (iii) cover the broad spectrum of reaction severity; (iv) recognise that a reaction can involve all organ systems yet still range in severity, and; (v) be validated as clinically useful. Development of such an ideal system is hampered by the lack of a "gold standard" of reaction severity against

Table 1.10: Grading system for sting anaphylaxis according to Golden et al[206]

Grade	Features
Minimal	Not specified
Skin	Generalised urticaria or angioedema only
Respiratory	Dyspnoea, throat tightness, asthma, cough
Hypotension	Dizziness, unconsciousness, blood pressure < 90/60 mmHg

Table 1.11: Grading system for colloid-induced anaphylactoid reactions according to Ring and Messner[207,208]

Grade	Skin	Gastrointestinal	Respiratory	Cardiovascular
I	Pruritis, urticaria, flushing	-	-	-
II	Pruritis, urticaria, flushing	Nausea	Dyspnoea, rhinorrhoea	Tachycardia, hypotension
III	Pruritis, urticaria, flushing	Vomiting, incontinence	Bronchospasm, cyanosis	Loss of consciousness
IV	Pruritis, urticaria, flushing	Vomiting, incontinence	Respiratory arrest	Cardiac arrest

Table 1.12: Grading system for insect sting anaphylaxis; systemic reaction index (SRI) according to Lockey et al[209]

Feature	Score	Grade
Unconsciousness	0.376	Severe (SRI 0.126 – 1.000)
Shock	0.376	
Drop in blood pressure	0.126	
Lower airway obstruction	0.050	Moderate (SRI 0.013 – 0.122)
Upper airway obstruction	0.050	
GI symptoms	0.013	
Angioedema/urticaria	0.003	Mild (SRI 0.003 - 0.009)
Pruritus	0.003	
Other	0.003	
Maximum total score (SRI)	1.000	

which the usefulness of each individual reaction feature and various grading systems might be judged.

Data with regard to the clinical utility of these grading systems comes from retrospective studies of accidental field re-stings using Reisman's system,[38] and deliberate sting challenge studies using the adaptation of Mueller's system described above.[52,198] These have demonstrated that prior worst reaction grades tend to predict subsequent reaction grades. Van der Linden and colleagues also demonstrated that the occurrence of a grade IV reaction (defined as per Table 1.9) is associated with higher plasma tryptase and histamine levels; mediator levels also correlate well with the magnitude of mean blood pressure fall from baseline.[131]

It seems reasonable to assume that sustained shock and hypoxia due to respiratory compromise are indeed severe, and therefore that various degrees of compromise to the cardiovascular and respiratory systems, as used by grading systems, are a good measure of severity. Nevertheless, hypotension may be transient as endogenous compensatory mechanisms take effect,[40] and thus may either go unrecognised (leading to an inappropriately low assigned grade) or may be of little significance in those with adequate compensatory mechanisms (thus leading to an inappropriately high assigned grade). Similar problems can be anticipated for respiratory reactions, and emergency medical treatment may reduce apparent severity. Therefore, in a routine clinical setting these grades often may not reflect true reaction severity.

Finally, with regard to the ultimate measure of clinical utility of these grading systems (mortality risk), studies of insect allergy deaths are limited by their retrospective nature, inability to interview subjects, and natural history of fluctuating reaction severity. Therefore, although prior reaction grade may predict subsequent reaction grade, whether prior "severe" reaction grades carry a higher risk of death is difficult to prove. One series of deaths identified five people where it was possible to get an indication of prior reaction grade; three had probably experienced hypotensive reactions (unconsciousness), one had experienced severe urticaria, and one was recorded simply to have been "unwell".[65]

1.4 Diagnosis of venom allergy

Current tests used to diagnose venom allergy examine for the presence of antigen-specific IgE (sIgE) in serum or the downstream results of surface sIgE activation

(clinical wheal and flare after injection of venom into the skin, basophil membrane activation marker expression, and leukocyte or whole blood mediator release).

1.4.1 Serum sIgE analysis

Current sIgE assays are derived from the radioallergosorbent test (RAST), which used antigen bound to a sephadex solid phase and was introduced by Wide, Bennich and Johannson in 1967.[210] Key steps are initial binding of antigen to the solid phase, followed by incubation with patient serum, washing, a second incubation with radio labelled anti-IgE and a final wash. Uptake of the radioactive tracer to the disc is then measured along with uptakes to positive and negative serum controls. Subsequent adaptations have included the use of cyanogens to couple soluble antigens to paper discs,[211,212] the use of dialysed venoms to improve sIgE binding,[213] and a method of binding both soluble and insoluble antigens to nitrocellulose discs without the use of cyanogens.[214] RAST was first applied to the diagnosis of stinging insect allergy in 1975.[215]

Because there is not a linear relationship between the amount of sIgE and radiotracer uptake and because subtle differences or interactions between minor and major allergenic determinants and their corresponding IgE antibodies may influence uptake,[216] RAST results are traditionally expressed as a semi-quantitative class. These are based on the percentage uptake of tracer (<2% = class 0; 2-5% = class I; 6-10% = class II; 11-20% = class III; ≥21% = class IV). Phadebas RAST methodology allows for comparison of IgE uptake to allergen-bound paper discs against known standards and expressed quantitatively in kU/L.[217]

The application of RAST testing has been limited by the effect of non-specific binding to allergen discs whereby the “signal to noise” ratio prevents detection of low levels of sIgE. This has resulted in lower sensitivity when compared to skin testing.[217]

Recently, the Pharmacia CAP System™ has been introduced that uses an encapsulated cyanogen bromide (CNBr) activated cellulose derivative, with higher antigen binding capacity than paper discs, improved reaction characteristics, and less non-specific binding during the assay procedure. Automated analysis for serum IgE uses radiolabeled anti-IgE and generates a quantitative result in kU/L. The CAP system is superior to RAST and may have a sensitivity equivalent to that of intradermal venom skin testing (97% for honeybee and 86% for vespid allergy).[218,219] Some false negatives may occur if testing is performed within a week or two of a sting reaction, with conversion to a positive result over the following two weeks.[220] Other factors such

as anti-IgE IgG auto antibodies may also influence quantitative measurements as well as sensitivity and specificity (see 1.5.8.2 below).

1.4.2 Venom skin testing

It is currently recommended that intradermal venom skin tests (VST), involving the injection of 0.02-0.03 ml of solution (enough to raise a small bleb), start at a venom concentration of between 0.001 and 0.01 ug/mL (mg/L). If initially negative, the concentration is increased tenfold until either a positive response occurs or a maximum concentration of 1.0 ug/mL is reached. Positive (histamine) and negative (diluent) controls are also required for comparison.[221] VST is considered safe; there have been no deaths reported; systemic reactions occur in 1.4% and are severe as defined by Lockey's grading system (Table 1.12) in only 0.25% of people tested.[222] One study has found elevations in serum sIgE reactivity by RAST to occur after skin testing in 50% of those tested, including conversion from negative to positive RAST.[223] However, an increased risk of clinical sensitisation has not been identified; in a large epidemiological study none of 120 persons exposed to skin testing who had an initially negative result went on to have a systemic reaction to subsequent stings.[192]

The methodology and definition of positive responses in published studies varies considerably (Table 1.13), making analysis of skin testing sensitivity and specificity difficult. Nevertheless, a clear pattern is apparent; namely, that sensitivity and specificity are determined by the venom concentration used and the insect venom in question. Overall, using prior reaction history as the diagnostic gold standard, the sensitivity for VST is 506/621 (0.81) using a venom concentration of 1.0 μg/mL, and 224/304 (0.74) using a venom concentration of 0.1 μg/mL. Overall, the specificity for VST is 356/446 (0.80) using a venom concentration of 1.0 μg/mL and 140/143 (0.98) using a venom concentration of 0.1 μg/mL. Studies including vespid wasp allergy report lower sensitivities than those studying honeybee allergy only, with the lowest sensitivity (0.68 using a venom concentration of 1.0 μg/mL) coming from a study of vespid wasp (yellow jacket) allergy. A direct comparison of honeybee and vespid wasp allergies has found that negative skin testing and RAST results are more prevalent with vespid allergy (80-86% sensitivity using a history of systemic reactions as the gold standard), whereas sensitivity of IgE analysis by VST or RAST is around 97% for honeybee sting allergy.[219]

Only 25-50% of those with detectable sIgE or skin test reactivity and a history of allergic reactions are indeed allergic according to sting challenge studies.[52,198] Of those with a history of allergic reactions who are skin test negative, a number go on to have allergic reactions when stung again. In one large "real life" study, only 2189 of 2880

Table 1.13: Intradermal venom skin test studies reporting both an allergic group defined by a history of systemic reaction to a sting, and/or a non-allergic control group

Study	Insect species & intradermal injection method	Conc	Allergic		Controls		Sens.	Spec.
			TP	FN	TN	FP		
Hunt[224]	**HB, V, P:** 0.05 mL injection; positive defined by wheal >5 mm and erythema >10 mm	1.0	30	0	29	1	1.0	0.97
		0.1	23	7	30	0	0.77	1.0
Miyachi[225]	**HB:** 0.05 mL injection; positive defined by wheal larger than saline control and either larger than 10mm, or surrounded by erythema	1.0	-	-	5	2	-	0.71
		0.1	30	4	12	0	0.88	1.0
Patrizzi[226]	**HB:** 0.01 mL injection; positive defined by wheal >5mm plus any degree of erythema	1.0	33	0	25	10	1.0	0.71
		0.1	31	2	34	1	0.94	0.97
Meriney[227]	**V:** 0.01 mL injection; positive defined by wheal >5mm plus surrounding erythema	1.0	19	1	7	3	0.95	0.70
		0.1	13	7	10	0	0.65	1.0
Wuthrich[228]	**HB & V:** 0.01 mL injection; positive defined by wheal &/or erythema >5mm	1.0	78	6	38	2	0.93	0.95
Harries[229]	**HB & V:** 0.05 mL; definition of a positive result not given	1.0	102	0			1.0	
		0.1	58	44			0.56	
Georgitis[230]	**HB, V & P:** 0.02 mL injection; positive defined by wheal >5 mm and erythema >20 mm after 15 minutes	1.0	76	9	30	26	0.89	0.54
		0.1	69	16	54	2	0.81	0.96
Golden 1989[188]	**HB & V (mainly V):** 0.02 mL injection to form a 3-4 mm bleb; positive defined at 20 minutes by wheal >5mm plus erythema >10mm	1.0	-	-	222	46	-	0.83
Golden 2001[107] *	**Almost exclusively V:** Injection volume and positive test definitions not specified	1.0	208	99	-	-	0.68*	-

HB- honeybee; V- Vespinae (vespid wasps); P- Polistinae (paper wasps); Conc- Concentration of venom solution in ug/mL (g/L); TP- true positive; FN- false negative; TN- true negative; FP- false positive; Sens- sensitivity = the likelihood that a person with the disease will return a positive result = TP/(TP + FN); Spec- specificity = the likelihood that a person without the disease will return a negative result = TN/(TN + FP).

For all the figures given above, allergic and non-allergic status was defined by the (retrospective) clinical history.

*In the Golden (2001) study, sensitivity of skin-test negative individuals was further examined by sting challenge, where 11/51 (22%) of skin test negative people and 30/141 (21%) skin test positive people reacted to sting challenge, giving sensitivity 0.73 and specificity 0.19. The results of this study were not entirely consistent with previous data, in that RAST appeared to detect a large number of cases missed by skin testing; some concern has since been expressed over the quality of venom extracts available for skin testing.[108]

people with a positive history (76%) had positive skin tests.[222] A study by Golden et. al. of 307 supposedly vespid wasp (yellow jacket) allergic people has addressed this problem using deliberate sting challenges in skin test negative individuals.[107] However, of 56 people who were both skin test and RAST negative only 14 were challenged,

selected "because of a more convincing or recent reaction history". Two of these 14 (14%) reacted to sting challenge, but this may be an inaccurate estimate of reaction risk in people with negative skin test and negative RAST because of the potential for selection bias in the absence of clear criteria for selecting these people for challenge. It should be noted that the confidence intervals for this figure are wide, and that there may have been problems with the venom extracts because skin testing had unusually poor sensitivity compared to RAST. Also, these results may not apply to honeybee or other insect allergies.

Multiple reactivity due to antibody cross-reactivity (or less commonly, multiple sensitisations) can be a significant problem in up to 50% of patients, making identification of the causative insect difficult in the absence of a clear history. This may be due to carbohydrate-specific IgE rather than IgE directed towards "major" antigenic determinants.[231]

VST and RAST appear to be of value in identifying the likely causative insect following a systemic allergic reaction. However, beyond this they appear to be of little use in diagnosing subsequent reaction risk. There appears to be no correlation between RAST class or skin test sensitivity and reaction severity.[52] Also, other factors (age and prior reaction severity) influence pre-test probability and must be taken into account. Thus, an elderly man with a history of grade IV (hypotensive) reactions would have a high pre-test probability. Therefore, VST and RAST may have insufficient negative predictive value to deny appropriate management on the basis of a negative result. Whether "appropriate management" should include immunotherapy in this situation is unknown; immunotherapy studies, discussed under 1.5 below, have been performed only in patients having clear evidence of sIgE reactivity.

1.4.3 In-vitro tests of leukocyte reactivity to venom

Measurement of mediator release by blood leukocytes (predominantly basophils) in response to incubation with venom was first described in the 1970s when it was reported that the **whole-blood histamine release test (HRT)** correlates reasonably well with skin test sensitivity.[224,232] Later research suggested that the HRT might be used to identify loss of reactivity, and thus might be more specific than skin testing or RAST.[233] More recently, two additional tests of venom-reactivity (sulphidoleukotriene generation and basophil CD63 expression) have been developed.

The leukotriene release test (LRT) uses ELISA to measure sulphidoleukotriene LTC_4 produced by isolated blood leukocytes in response to incubation with venom. When using the "gold standard" of a positive venom skin test, a higher sensitivity

and specificity of LRT compared to HRT has been reported in a study of 23 honeybee and 100 vespid wasp allergic subjects; sensitivity was 100% for honey honeybee venom allergy, 83% for vespid venom allergy and specificity was 77% for both venom allergies.[234] This study used the proprietary Cellular Antigen Stimulation Test (CAST™), where leukocytes are primed with IL-3 prior to incubation with venom to maximise their reactivity in terms of mediator production, and where the ELISA monoclonal antibody is directed at LTC_4 and its metabolites LTD_4 and LTE_4.

The basophil activation test (BAT) uses double antibody flow cytometry analysis to detect CD63 positive basophils after incubation with venom (usually associated with IL-3 priming). Antibodies are directed against IgE (e.g. anti-IgE FITC) and CD63 (e.g. anti-CD63 PE). Results are expressed as the percentage of basophils positive for CD63, a glycoprotein present on the lysosome membrane of various cell types. When degranulation occurs this glycoprotein is incorporated into the cell membrane and is therefore a marker of degranulation. 100% sensitivity and 100% specificity have been claimed for the diagnosis of venom allergy by BAT.[235] However, allergic individuals were defined by "clinical diagnosis on the basis of skin test reactivity, history, serum sIgE and histamine release", people with large local reactions without systemic reactivity were designated as "venom allergic", and VST were not performed on non-allergic controls.

No studies have assessed these *in vitro* techniques against the best available gold standard of clinical reactivity –sting challenge combined with prospective follow up– in a clinically relevant group of people (i.e., people with a history of systemic allergic reactions presenting for assessment).

1.4.4 Deliberate sting challenge

Given the poor specificity (and imperfect sensitivity) of skin tests, the infrequency of accidental sting exposures and the problems inherent in interpreting the results of an unobserved field sting, deliberate sting challenge remains the gold standard for determining the presence of clinical reactivity to insect venom. Even so there remain significant problems.

Two studies have examined outcomes of people with a history of anaphylaxis and positive skin tests or RAST, who were not given immunotherapy based on a negative sting challenge result. In a follow-up of 327 such people with a history of mainly vespid wasp sting allergy, 129 were accidentally re-stung; 13 (10%) reported mild systemic symptoms; 6 (5%) reported serious manifestations including 2 with documented hypotension.[204] Another study confined to people with a history of vespid

wasp allergy, submitted 61 people with an initially negative sting challenge result to a second deliberate sting challenge; 13 (21%) had a systemic reaction including 6 with hypotension (compared to a 40% reaction rate to the initial sting challenge).[236] Some of this variability may be due to problems with Vespid sting challenges, as these wasps tend to inject highly variable amounts of venom,[199] and during handling may "spray" a significant amount of venom before patient contact.[237]

It is unclear from these studies whether people with a history of severe reactions have a higher risk of reacting to subsequent stings despite an initially negative sting challenge result. Interestingly, people without a history of sting anaphylaxis but positive skin tests have been found to have a similar reaction rate (17%) to subsequent field stings.[192] Thus, a negative sting challenge result may simply indicate a reaction risk similar to any person with demonstrable sIgE yet without prior systemic reactions.

1.5 Specific immunotherapy

The first recorded attempts at specific allergen immunotherapy (SIT), utilising the injection of gradually increasing doses of allergen, starting with extremely dilute concentrations, were for the treatment of hay fever in 1911. At that time hay fever was thought to be the result of direct pollen toxicity in individuals with a "idiosyncratic sensitivity", and that "prophylactic inoculation" with pollen extracts would induce protective antibodies.[238,239]

Early experimentation with venom immunotherapy was hampered by difficulties extracting suitable amounts of venom. In 1925, Braun reported a case of successful immunotherapy using extract from the "rear eighth" of honeybee gasters on a beekeeper,[137] and in 1930 Benson reported the successful use of a mixture of "bee stinger protein" and "body protein".[138] Body protein was considered a necessary component on the basis of patient skin test reactivity to various extracts. Whole body extracts (WBE) subsequently came to be applied worldwide to treat allergy to many stinging and biting insects.

From 1959 onwards, a number of retrospective studies claimed success of honeybee and vespid WBE treatment, leading to statements such as "there can no longer be any question of the efficacy of desensitisation for allergy to insect sting".[42] Analyses of WBE and venom sacs even resulted in the conclusion that "the antigen causing reactions is present throughout the insects body, but the venom contains a small amount of antigen".[35] However, with improved analytical techniques reports of absent or low

levels of venom allergen in WBE extracts soon emerged,[240,241] and a number of patients died from stings despite WBE immunotherapy.[98,242,243] Simultaneously, techniques for producing large amounts of pure venom extract by electrical stimulation were improved, beginning with Benton's description of a device for large scale milking of venom from honeybees.[244] Two controlled studies then found WBE treatment to be ineffective whilst pure venom immunotherapy (VIT) appeared to be a highly effective treatment for honeybee and wasp sting allergy.[245,246]

In the USA, imported fire ant (IFA) venom allergy continues to be managed with the use of WBE. Because of the continued use of WBE for IFA allergy, the evidence for efficacy of both WBE and pure venom extracts will be reviewed in detail.

1.5.1 Evidence for clinical efficacy

1.5.1.1 Whole body extract (WBE) immunotherapy

A summary of published studies of immunotherapy using insect WBE is presented in Table 1.14. Earlier studies were hampered by retrospective designs predisposing to recall and selection biases, and reliance on accidental stings where insects were not positively identified and reactions were not objectively monitored. Most studies used the outcome measure of sting reactions being "less severe" after treatment, an outcome that is predictable by the natural history of insect sting allergy.[52,256] Also, variable amounts of venom may be injected by a sting,[109,199] which may explain the observation that reaction severity can fluctuate over time.[34] Finally, in almost all studies, there was absent or poor reporting of inclusion criteria, exclusion criteria, and whether (or why) some subjects were lost to follow-up.

In addition to these general methodological limitations, Mueller's 1959 study[35] is also affected by the predominance of children allergic to wasp stings, resulting in a study group with a very low subsequent reaction rate even without treatment because of the tendency for children to loose reactivity,[197,256] and because wasp sting allergy has a low re-sting reaction rate.[52,198]

In the largest study, by the Insect Allergy Subcommittee,[37] it appeared that the natural history of untreated insect allergy was for only 9.4% of people to have less severe reactions when stung again- a result that was probably due to selection bias (people with recurrent severe reactions being more likely to come forward). However, during the prospective study almost 40% of untreated patient appeared to loose reactivity. The majority of those treated with WBE had "less severe" reactions during the prospective

study period, however the very high adrenaline administration rates suggest that this subjective improvement was due to early treatment with adrenaline.

The only studies to employ prospective methodologies with matched control groups appear to demonstrate that WBE therapy for honeybee and wasp allergy is ineffective.[245,246] Why were clinicians apparently mislead for so long? Although the scientific methods commonly utilised in clinical investigations of the time were inadequate, it is possible that WBE treatments were effective in some individuals. Some investigators used extracts from the "terminal eighth of the gaster", or supplemented by additional stinger mechanism proteins,[138] and freshly prepared extracts may have included significant amounts of venom. Thus, the reason for the dramatic failures of WBE when later tested against venom extracts may be the poor quality of commercially supplied WBE.

A study comparing commercial honeybee and wasp extracts available in 1979 with freshly prepared extracts found no detectable venom activity in commercial extracts, however venom activity was present in freshly prepared extracts, even though the later were made from the whole body,[257] (that is, whole body not restricted to the terminal gaster or supplemented with venom protein as described above). Further evidence supporting the hypothesis that some extracts may have contained therapeutic amounts of venom allergen can be found in early descriptions of allergic reactions, some requiring "heroic resuscitation", to very low doses of WBE.[35]

WBE continues to be advocated for the treatment of IFA allergy. Some commercially available IFA extracts contain venom activity.[258-260] The evidence supporting clinical efficacy includes one large case series employing deliberate sting challenges,[255] and a retrospective study using accidental and deliberate stings in WBE treated patients compared with accidental stings in a small control group of people who chose not to be treated.[254]

Although promising, these IFA studies have significant limitations. In addition to the methodological flaws outlined in the section above, it was not shown that the deliberate sting methodology employed (a single sting repeated after 2 hours) could induce severe anaphylaxis and thus provide a useful test of treatment efficacy. A single fire ant sting delivers only 10 to 100 ng of venom[261] and attacks usually involve multiple ants, with 7-8 stings per ant.[262] Furthermore, the natural history of IFA sting allergy remains poorly defined, without any prospective longitudinal studies of untreated allergic people. Variable allergen contents of commercially available IFA extracts have

Table 1.14: Studies of insect whole body extract (WBE) immunotherapy efficacy

Author	Insect	Patient Population	Study Design	Enrolled	Sting	Results
Mueller[35] (1959)	Unclear*	Mainly children, milder allergy	Uncontrolled analysis of people receiving WBE	78	A	30 stung: SR in 5/30 (17%); 1 to different insect, 1 before reaching maintenance; remaining 3 "less severe".†
Frazier (1964)[42]	H, W	All ages and severities	Uncontrolled analysis of people receiving WBE	Unclear	A	41 stung; reactions "less severe" in 38, "more severe" in 2.
Insect Allergy Committee, AAA (1965)[37]	H, W	All ages and severities	Retrospective survey followed by prospective study comparing WBE with no treatment; no reasons given for treatment choice	2606	A	**Retrospective:** SR "less severe" in 580/647 (90%) after WBE, versus 72/763 (9.4%) in those stung a second time before WBE. **Prospective**: SR "less severe" in 232/261 (89%) after WBE versus 40/102 (39%) in untreated group. *However, adrenaline administration rates for sting reactions were 38% versus 15% respectively.*
Wortmann (1969)[247]	H, W	All ages and severities	Uncontrolled analysis of people receiving WBE	30	A	15 stung; 3 (20%) experienced "transient dizziness".
Brown (1970)[34]	H, W	All ages and severities	Comparison of treatment (WBE or venom), with no treatment; allocated no treatment if negative ST	400	A	103 stung: SR in 4/61 (7%) after therapy, versus 3/42 (7%) in no treatment group. Unclear as to how many of those stung had received venom rather than WBE. Variable SR severity in many people over time.
Triplett (1973)[248]	IFA	All ages and severities	Uncontrolled analysis of people receiving WBE	18	A	8 stung: SR in 0/8.
Hoigne (1974)[249]	H, W	All severities, ages not stated	Uncontrolled analysis of people receiving WBE	61	A	24 stung; SR in 8 (33%), "less severe" in 5.
Mueller (1975)[250]	Unclear*	Mainly children, milder allergy	Uncontrolled analysis of WBE treated people; only 400 of 700 contactable	400	A	217 stung while receiving treatment; SR in 11 (5%).†
Rhoades (1975 and 1977)[251,252]	IFA	Adults, all severities	Uncontrolled analysis of people receiving WBE	92	A	19 stung; SR in 2 (11%).

Wuthrich (1977)[253]	H, W	All ages and severities	Uncontrolled analysis of people receiving WBE	123	A	54 stung; SR as severe as prior reactions in 13 (24%).
Hunt (1978)[245]	H, W	Adults, all severities	Single blind placebo *vs* WBE *vs* VIT; not randomised‡	19 VIT; 20 WBE; 20 placebo	STC	1 withdrawn from VIT (side effects). SR in 7/12 (58%), 7/11 (64%) and 1/18 (5.5%) in placebo, WBE and VIT groups respectively.
Müller (1979)[246]	H	All ages and severities	Open label comparison VIT with WBE, arbitrary randomisation	31 VIT; 25 WBE	A	24 stung: SR 3/12 (25%) and 9/12 (75%) in VIT and WBE groups respectively.
Freeman (1992)[254]	IFA	All ages and severities	Retrospective study of people "currently followed", comparing those choosing WBE versus no WBE	65 WBE; 11 no treatment	A + STC	WBE group had lower incidence of severe allergy and included children. There were no children in the untreated group. **Accidental stings:** SR in 1/47 (2.1%) and 6/6 (100%) in WBE and no treatment groups respectively. **Sting challenges:** No SR in 0/30 patients receiving WBE.
Tankersley (2002)[255]	IFA	Adults, all severities	Uncontrolled prospective study of rush WBE therapy	59	STC	Systemic reaction to sting challenge in 1/56 (1.7%).

H = honeybee; W = wasps (predominantly yellow jacket vespid); IFA = imported fire ant; ST = skin test; SR = systemic reaction; A = accidental field sting; STC = deliberate sting challenge.

Studies of <10 patients and others reporting very limited patient details have been excluded from analysis.[3,36] *Mix of species involved not stated as mixed (4-genera) WBE used in treatment, but from earlier publications by Mueller it appears that the vast majority of his cases had reacted to yellow jacket (vespid) stings. †It is interesting to note that Mueller reported systemic allergic reactions to WBE immunotherapy, as well as observing that the rate of systemic reactions during immunotherapy was less if a higher maintenance dose was used; this suggests that the extract used in his practice may have contained significant amounts of venom protein. ‡Subjects were allocated to treatment groups arbitrarily to produce subjectively "matched groups" in terms of skin reactivity, reaction severity and in-vitro test results

been reported,[260] along with reports that WBE are not as good as venom for diagnostic use,[263,264] and treatment failures have been reported.[265,266]

M pilosula WBE

No studies of venom antigen content of the *Myrmecia* spp. WBE produced by CSL were ever published. However, *Myrmecia* spp WBE preparations were withdrawn in the early 1990s and at a scientific meeting in 1995 were reported to be ineffective on the basis of a retrospective analysis of field stings in WBE treated individuals.[3] Because of the paucity of clinical data reported by this study, it has not been included in Table 1.14.

1.5.1.2 Venom immunotherapy (VIT)

A summary of published studies of VIT including 10 or more patients and assessment of efficacy by either sting challenge or accidental sting is presented in Table 1.15. A disturbing feature is that systemic sting reaction rates of 21-42% after VIT have been reported by no less than six groups,[246,269,270,272,274,281] a reaction rate equivalent to that observed in sting challenge studies of un-treated subjects.[52,198] The argument is frequently made that most reacting patients still constitute "treatment successes" because reactions are less severe after immunotherapy, however this is also a feature of the natural history of sting allergy.[52]

Varying immunotherapy regimes, timing of sting challenge, reliance on field versus deliberate stings, and differences in pre-treatment risk to patients between studies make comparisons and conclusions difficult. Overall failure rates, as defined by any degree of systemic reaction following a sting, are higher for studies of predominantly honeybee venom allergic patients (56/272, 18%) than for studies enrolling mainly vespid venom allergic patients (12/255, 4%). Honeybee venom allergic patients also have higher skin test reactivity and higher RAST scores, and develop side-effects to VIT more often than those allergic to *Vespula* (yellow jacket) venoms.[283] These findings indicate that studies of allergy to one Hymenoptera cannot be extrapolated to other species. Studies in children with milder degrees of allergy report lower VIT failure rates (0-3%)[197,256,267] whereas the highest failure rate has been reported by a study mainly of adults with prior sting reactions characterized by severe bronchospasm and using an unusual VIT technique (periodic rush desensitisation rather than regular maintenance doses).[269]

These differences between studies reinforce the need for a concurrent untreated control group with proper randomisation and sufficient numbers of participants to ensure that treatment and control groups are properly matched in terms of age, prior

reaction severity and perhaps other as yet unknown predictors of reaction risk. No published study fulfils these criteria. Nevertheless, the pooled reaction rates following VIT are less than the untreated reaction risks determined by the large sting challenge studies (21% versus 50% for honeybee, and 5% versus 20% for wasp, 13% versus 27% overall). Furthermore despite their methodological limitations, studies that have attempted to provide untreated (or WBE treated), evenly matched control groups have found VIT to reduce reaction risk substantially.[197,245,246,256]

Consensus statements recommend VIT be offered to people with a history of systemic reactions, however recommendations vary regarding the severity of reactions required to justify the inconvenience and cost of therapy. Whereas North American guidelines list any degree of systemic reactivity as an indication for VIT (except in children with skin-only reactions for whom treatment is not recommended),[221] a world health organization position paper lists reactions with cardiorespiratory features as an absolute indication and lesser reaction severities as a relative indication for VIT.[284] This rationale is based on observations that people with less severe reactions rarely progress to have more severe reactions (see 1.2.7 above). In one large multi centre North American study, only 64% of eligible people (skin test positive plus any degree of systemic reactivity) elected to receive VIT.[209] Whether the decision to undergo treatment was influenced by prior reaction severity was not reported.

Several difficulties can lead to sub optimal outcomes outside of the idealized clinical trial setting. Firstly, the causative insect and thus appropriate treatment can be difficult to identify when skin testing &/or RAST reveal multiple reactivities. Secondly, poor tolerance of VIT due either to the inconvenience of multiple visits or adverse reactions may lead to the patient withdrawing from treatment or inability to achieve an adequate maintenance dose (see 1.5.3 below). Finally, many patients with a clear reaction history may have negative skin tests. A staggering 120/183 (66%) of patients with a history of systemic reactions were excluded from Hunt's landmark trial of VIT on the basis of negative skin tests.[245] Recent work by the same group, using modern venom preparations and performed in the setting of predominantly *Vespula* (yellow jacket) venom allergy, found that 99/307 (32%) of people with positive reaction histories to be skin test negative; 56/307 (18%) were both skin test and RAST negative. No trials have been performed to examine the efficacy of VIT in this group of patients.[107] As discussed in 1.4.2 above, this may be less of a problem for honeybee sting allergy where the sensitivities of skin testing and RAST appear to be in the order of 97%.

Table 1.15: Studies of venom immunotherapy (VIT) efficacy

Author	Population	Insect	VIT type	MD (ug)	Study design	Enrolled	Unable to tolerate VIT	Sting	Reaction rates to sting
Hunt (1978)[245]	Adults, all severities	Mostly W	MR	100	Single blind placebo *vs* WBE *vs* VIT; not randomised*	VIT 19; WBE 20; placebo 20	1	STC	VIT 1/18 (6%); WBE 7/11 (64%); placebo 7/12 (58%)
Urbanek (1978)[267]	Children, all severities	H	R	100	Uncontrolled analysis of VIT	11	0	STC	0/11 (0%)
Müller (1979)[246]	All ages, all severities	H	MR and R	80-200	Open label VIT *vs* WBE, arbitrary allocation	VIT 31; WBE 25	NS	A	VIT 3/12 (25%); WBE 9/12 (75%)
Abkiewicz (1979)[268]	All ages, all severities	H	MR	100	Uncontrolled analysis of VIT	40	NS	A	0/11 (0%)
Yunginger (1979)[269]	All ages, severe	H	R†	200	Uncontrolled analysis of VIT	20	1	STC	8/19 (42%)†
Gillman (1980)[270]	All ages, severe	Mostly H	MR	100	Uncontrolled analysis of VIT	23	0	STC	4/19 (21%)
Golden (1980)[271]	Adults, all severities	Mostly W	C, MR, R	100	Comparison of C, MR and R VIT regimes	64	2	STC	0/52(0%) (excluded those unable to tolerate VIT)
Golden (1981)[272]	Adults, all severities	Mostly W	MR	50	Uncontrolled analysis of VIT	23§	0	STC	4/19 (21%)
Clayton (1983)[273]	All ages, severity NS	NS	C & MR	50	Uncontrolled analysis of VIT	31	NS	A	1/12 (8%)
Thurnheer (1983)[274]	Adults, all severities	H & W	C & R	100	Comparison of conventional and rush immunotherapies‖	42	1	A	7/23 (30%)
Peppe (1983)[275]	All ages and severities	H & W	NS	NS	Multi-centre observational study; VIT vs no VIT	2701	NS	A	VIT 42/407(10%); no VIT 11/117(9%)

Nataf (1984)[276]	Adults, severe	Mostly W	R	100-200	Uncontrolled analysis of VIT¶	52	NS	STC	0/14 (0%)
Malling (1985)[277]	Adults, all severities	W	MR	200	Randomised comparison of two MR regimes	25	4	STC	0/16 (0%)
Reisman (1985)[278]	Adults, all severities	H & W	C or R	50	Stings with VIT *vs* after stopping VIT, *vs* no VIT	With 127; post 56; none 88	NS	A	With 1/48(2%); post 11/41 (27%); none 14/28 (50%)
Mosbech (1986)[279]	Adults, all severities	W	C	100	2.5 yrs Rx with dialysed *vs* Al-absorbed venom	32	2	STC	0/19 (0%)
Adolph (1986)[280]	Ages NS, all severities	Mostly H	R	100	Uncontrolled analysis of VIT	208	NS	A	7/52 (13%)
Przybilla (1987)[281]	All ages and severities	H & W	R	100	Uncontrolled analysis of VIT	290	1	STC	33/157 (21%)
Bousquet (1989)[282]	All ages and severities	H & W	R or MR	200	Uncontrolled analysis of VIT	200	NS	A	5/76 (7%)
Schuberth (1983) and Valentine (1990)[197,256] **	Children, mild allergy	H & W	C	100	Prospective, open, partly randomised**	VIT 68; no treatment 174	NS	A	VIT 1/36 (3%); no treatment 16/86 (19%)
Müller (1992)[283]	Adults, all severities	H & W	R or C	100	Comparison of H and W VIT	H 148; W 57	NS	STC	H 34/148 (23%); W 5/57 (9%)

SR = systemic reaction; H = honeybee; W = wasp (mainly yellow jacket vespid); C = Conventional (single injections weekly until achieving maintenance dose); MR = “Modified”, “rapid” or “semi-rush” (2 or more injections weekly until achieving maintenance dose); R = Admission for “rush” immunotherapy (multiple injections over several days, followed by weekly-monthly injections); NS = not stated.

Studies of <10 patients and others reporting only limited patient details have been excluded from analysis. *Subjects were allocated to treatment groups arbitrarily to produce subjectively “matched groups” in terms of skin reactivity, reaction severity and in-vitro test results †An unusual rush schedule was used (admitted for clusters of rush VIT up to a month apart), and up to 4 stings were administered during the day of sting challenge. §This group, receiving 50 ug maintenance doses, was compared with patients receiving 100 ug (either previously reported or presented without any clinical details and therefore not included in this analysis). ‖Patients recruited in summer were allocated R VIT and those recruited in winter were allocated C VIT. ¶Primary outcome measure was tolerance of immunotherapy. Method of selecting for sting challenge not stated. **Majority of cases appear to have been reported twice in these papers, but this is not clearly stated. Participants were given a choice between randomisation and selecting their own treatment; groups appeared to be well balanced with regard to baseline characteristics and of those who chose for themselves, the majority chose no treatment.

1.5.2 Initiation phase

Several approaches to initiating VIT have been published (Table 1.15). Starting doses are in the region of 0.0001mcg, gradually increasing to a maintenance dose of 100mcg. Conventional VIT involves the administration of a single dose each week, gradually increasing the dose until maintenance is achieved then extending the dosing interval out to monthly. Rush VIT involves admission for multiple injections with the aim of achieving maintenance dose within several days. Several "cluster", "modified", "rapid", or "semi rush" approaches performed over months in an outpatient setting have been described whereby the common feature is administration each week of several doses over a number of hours. "Ultra-rush" techniques have been described that compress the initiation phase into 2 days,[285] 6 hours,[286] and 2½ hours.[287] In one of these studies comparing 4 day, 6 hour and 2½ hour initiation phases, lower reaction rates were seen in the 2 ½ hour group, in which a lower cumulative dose was given. Patients receiving bee venoms also reacted more often than those receiving yellow jacket venoms.[287]

The target maintenance dose is arbitrarily defined. 100mcg was initially used on the basis that this is an amount of venom comparable to two honeybee stings (or several wasp stings) and subsequent comparisons with a lesser dose of 50mcg indicated significantly lower efficacy with the later dose.[272] One study of patients who were treatment failures at 100mcg (28% of whom had elevated baseline serum tryptase levels) has claimed success using higher doses (150-400 mcg).[201]

1.5.3 Safety, tolerability and compliance with immunotherapy

Although deaths have been reported secondary to skin testing with food and aeroallergens and with aeroallergen immunotherapy, surveillance systems in the UK and USA have not identified any deaths due to intradermal venom skin testing or VIT.[288,289] However, both these studies were published within 8 years of the first trial of VIT and no data from nearly two decades of treatment since then have been presented.

In a multi centre North American study,[209] 12% experienced a mean of 1.9 systemic hypersensitivity reactions; 9% had these before reaching the 100ug maintenance dose (usually at doses between 1 and 50 ug), 2% had reaction(s) both before and after achieving maintenance, and 3% experienced their first reaction at maintenance. Approximately 1 in 10 reactions were classified as severe, as defined by Lockey's grading system (Table 1.12). Ninety-one percent achieved maintenance dose, 84% continued treatment after achieving maintenance, and 77% were still receiving VIT

at 3 years. Although only 23/297 (8%) of dropouts listed adverse reactions as the main reason for ceasing VIT, significantly more dropouts (overall number not stated) had experienced adverse reactions. Combined with the fact that only 64% of eligible patients chose to receive VIT in the first place, it can be seen that less than 50% of eligible people end up benefiting from VIT. It is apparent that the inconvenience and economic impact of immunotherapy, and sometimes-slow progress due to mild-moderate reactions have a major impact. Systemic reactions were more common in people undergoing honeybee (40%) and paper wasp (35%) than for vespid VIT (12%). Another large study of honeybee and vespid allergic patients treated in a single clinic has confirmed a higher systemic reaction rate in patients receiving honeybee VIT than for those receiving *Vespula* (yellow jacket) VIT (41% versus 25% respectively).[283]

Two studies have compared rush with outpatient (conventional and modified rush) schedules.[271,274] One compared conventional and rush therapy in mainly honeybee allergic patients, and the other compared conventional, modified rush and rush schedules in vespid allergic patients. Neither found any significant difference in the proportions of patients experiencing reactions between groups however numbers were small (~20 in each treatment arm in each study), both studies thus being grossly underpowered (only 55% power to detect a increased reaction rate of 25%; calculations performed using Power and Precision[290]). Combining the results of all published studies, the overall reaction rate for inpatient rush regimens is 26%, compared to 11% for outpatient conventional and clustered/modified rush regimes.[207]

The ability to avoid systemic reactions during any type of immunotherapy (conventional or rush) may be operator and experience dependant. Earlier studies reported systemic reactions in as many as 67% of patients, whereas a recent study using a 4-day rush regime reported reactions in only 7 of 100 patients.[207] One group has also reported a reduction in systemic reaction rates over time along with a gradual compression of the inpatient rush regime to a mere 2 days.[285] However, it is also possible that earlier studies were more likely to include highly allergic (and thus highly motivated) individuals. As a treatment becomes more widely available, studies may recruit more people with less severe disease.

1.5.4 Antihistamine pre-medication

In a number of prospective double-blind placebo controlled studies, pre-medication with H_1 blockers has been shown to reduce the incidence of systemic reactions and also severe local reactions during cluster, rush and ultra-rush immunotherapy,[291-294] whereas the addition of H_2 blockers provides no additional benefit.[294] In a 3-year, retrospective follow up of subjects enrolled in one of these studies of ultra-rush immunotherapy

for severe (Muller grade III-IV) honeybee sting allergy, Ulrich Müller's group found evidence suggesting H_1 blocker pre-medication may enhance the medium-term efficacy of treatment in terms of protection from sting reactions.[295] *In vitro* studies have indicated that histamine promotes the production of pro-allergic TH_2 cytokines IL-4 and IL-5 from T cells,[182] providing a plausible mechanism by which antihistamines may enhance the efficacy of VIT.

1.5.5 Maintenance dosing interval

Early studies of VIT arbitrarily chose a maintenance-dosing interval of 1 month. Subsequently this interval has been successfully increased to 6 weeks,[296] and three months,[297-299] using a combination of accidental stings and deliberate sting challenges to confirm efficacy which was around 95% in those able to tolerate the extended maintenance interval. Only mild reactions occurred in those who reacted to sting challenge. A large number of both honeybee and *Vespula* spp. venom allergic patients have been studied.[297-299] In the earliest study only 35/50 (70%) of honeybee versus 117/128 (91%) of *Vespula* spp. venom allergic patients were able to tolerate attempts to extend the maintenance interval beyond 1 month. About half of the remaining patients experienced systemic reactions on attempting to increase maintenance intervals in the remainder medical notes did not record the reasons for failing to extend the maintenance interval.[297] Unfortunately, baseline characteristics of the patient population, and any correlation between prior reaction severity and ability to extend the maintenance interval were not provided. The studies from Goldberg's group[299] included a very high proportion of people with very mild allergy and only 6 people (3.8%) were unable to achieve a 3-month maintenance interval (129 of 160 patients reported prior reactions of Mueller grade I and II severity).

These results suggest that although attempts to increase the maintenance interval to 3 months are appropriate (and efficacious if tolerated), a significant proportion (perhaps as many as 30% in some patient populations) may not tolerate such attempts. Although it might be expected that difficulties could be more likely in honeybee sting allergic people, and those who have a history of more severe reactions, the available data is inconclusive in this regard.

1.5.6 Duration of therapy

In studies of predominantly vespid allergic adults, there is a substantial risk of systemic reactions if accidentally stung after dropping out early from VIT. In two studies incorporating a total of 69 patients stung accidentally after discontinuing VIT against medical advice, a reaction rate of 17-22% has been observed.[278,300] Although less than

the 35% follow-up reaction rate observed in subjects choosing no treatment in one of these studies,[278] it is not significantly different from reaction rates observed during sting challenges of untreated vespid venom allergic patients.[52,198] An 8% reaction rate has been reported in vespid allergic patients in whom VIT has been ceased after returning "insignificant" RAST titres.[301] It should be noted however that many of these subjects were still positive to skin testing at 0.1 mcg/ml.

Golden's group has published a series of papers on cessation of VIT in predominantly *Vespula* spp. venom allergic patients with severe (respiratory or cardiovascular compromise) allergy.[206,302-304] When performing deliberate sting challenges 1 year after ceasing 5 or more years of VIT; no reactions occurred in 29/30 patients.[302] In a later study of 74 patients (where again, VIT had been continued for at least 5 years and stopped regardless of skin test or RAST reactivity) regular sting challenges were performed over a 4-year period. Ten percent experienced systemic reactions. All sting reactors had demonstrable sIgE at the time of cessation of VIT, but it was noted that this was frequently within a range not detectable by some commercial tests and associated with a negative intradermal skin test.[303] During follow ups of accidental stings in these and other clinic patients, 16/133 (12%) had systemic reactions- 4 were associated with respiratory or cardiovascular compromise (versus 50% of cases prior to commencing VIT). Half of the reactors had experienced a reaction during immunotherapy (to either the immunotherapy itself or to an accidental sting). A cumulative increase in reaction rates was noted with a 10% reaction risk for each sting such that in the original group of patients, the cumulative risk of a reaction after stopping VIT had climbed to 16%, appearing to be heading for a plateau of 20% after 10-20 years off-treatment.[206,304] This reaction rate is similar to that found in untreated *Vespula* spp. allergic people who are subjected to sting challenge.[52,198]

In an analysis of patients accidentally stung within 3-7 years after stopping VIT, Lerch and Müller found systemic reaction rates of 19/120 (16%) and 6/80 (8%) for honeybee and vespid allergic patients respectively. Patients experiencing systemic reactions were significantly more likely to have been treated with VIT for less than 5 years. Most reactions occurred after a first sting and were mild, however after repeated accidental stings systemic reactions tended to be severe. The authors state that a negative intradermal skin test at 1 mcg/mL at the cessation of immunotherapy appeared to be associated with protection from subsequent reactions. However, as the observed rate of reactions was inherently low and a negative skin test was evident in only 8 people no firm conclusion can be drawn from this study alone.[237]

In a study of children and adolescents with honeybee sting allergy, 31/66 demonstrated a substantial reduction in venom-specific RAST uptakes and therefore VIT was stopped after 3-4 years; 16 of these 31 children had previously experienced severe (Mueller grade III-IV) sting reactions. Twenty-nine were subjected to deliberate sting challenges at 1, 2 and 3 years after cessation of immunotherapy. Mild reactions (cough and conjunctivitis) occurred following sting challenge in 1/29 (3%) at 1 year and 2/29 (7%) at 2-3 years.[305] Golden's group also found that sIgE RAST uptakes were significantly higher in reactors versus non-reactors to sting challenges performed 2-4 years after cessation of immunotherapy.[303]

Three deaths have been reported following cessation of immunotherapy. One of these occurred in a 72-year-old man with a history of a hypotensive reaction following a honeybee sting, who received VIT for 4 years at the completion of which his skin test remained positive at 1 mcg/mL. He died after a honeybee sting 9 years later, apparently his first sting after stopping VIT.[306] The others occurred after cessation of VIT in two people with histories of "severe" reactions to yellow jackets and established diagnoses of mastocytosis; skin test sensitivity at the time of ceasing VIT was reported for only one these cases, who was positive at 0.01 mcg/mL.[307]

Current guidelines suggest that it is appropriate to cease immunotherapy after 5 years, although a residual risk, even in the presence of a negative skin test, is recognised.[221] It has been suggested that VIT should be continued indefinitely in those with a history of either a grade IV reaction prior to immunotherapy, or a reaction during immunotherapy (to either the immunotherapy or an accidental sting).[308]

1.5.7 Long term safety

Little data is available on the long-term safety of immunotherapy (adverse effects other than systemic allergic reactions), apart from the assumptions that the various groups following patients for many years would have detected unusual adverse events (see 1.5.6 above) and that the various national adverse reaction monitoring organizations would have detected a problem over the 20 or so years that VIT has been in widespread use. One study evaluating the safety of VIT in 66 children over a treatment period of 5 years with physical assessments and laboratory examinations found no evidence of long-term adverse effects.[309]

1.5.8 Mechanisms

Determining the mechanisms by which VIT works is complicated by the variety of VIT techniques (ultra-rush, rush, clustered/semi-rush and conventional) and different

time points at which measurements can be taken. Thus, at different time intervals and with different VIT techniques, different mechanisms may be evident.

Insights into the general mechanisms by which SIT has an effect have been gained from studies of immunological changes during immunotherapy with dust mite and pollen extracts. It is difficult to know how such aeroallergen SIT studies apply to the mechanisms of VIT and again, differing regimes complicate the analysis of results.

1.5.8.1 Clinical observations

As noted above, clinical experience indicates that although VIT may protect a majority from life-threatening reactions, in many people sting reactions continue to occur, albeit less severe than prior to treatment. Reactions to VIT itself occur after maintenance doses have been achieved; this is the first time that a systemic reaction to therapy occurs in around 36% of those experiencing reactions to VIT.[209] Thus, VIT appears to alter the pattern of mediator release in many individuals and may simply change the threshold allergen dose at which reactions occur (which may fluctuate from time to time according to maintenance intervals and other unknown factors), rather than causing true "desensitisation".

If true "desensitisation" to normal immune reactivity occurs in some individuals, we might expect to see a long-lasting effect that persists beyond the cessation of treatment. The ability of specific immunotherapy, including venom immunotherapy, to produce such a long-lasting reduction in sensitivity remains to be determined. One retrospective study of grass pollen SIT for rhinitis suggests that effects may be long lasting for at least 3 years after cessation of treatment.[310] However, long term VIT studies performed by Golden's group have found that vespid venom sensitivity redevelops after treatment cessation, with a prevalence of clinical sensitivity approaching that of untreated patients after 10-20 years (see 1.5.6 above).

Some data suggests that SIT may prevent the development of new sensitisations, thus suggesting a global (rather than allergen-specific) alteration in the balance between TH_1 and TH_2 response to antigens. An unblinded, non-randomised prospective study comparing house dust mite SIT with drug-only treatment for sensitised asthmatic children found that SIT was associated with a lower incidence of development of new sensitivities; 12/22 (55%) versus 22/22 (100%).[311] Another retrospective analysis of initially mono-sensitised adults with allergic rhinitis and/or asthma found that 3 years after completing a 4-year treatment course 1936/7182 (27%) had multiple sensitisations compared to 932/1214 (77%) in a control group of people choosing not to receive SIT.[312] Whether such benefits continue into the longer term remain to

be demonstrated, and further randomised controlled studies are required to confirm these observations. No comparable data is available as to whether VIT prevents the development of additional venom allergies.

1.5.8.2 Antibody changes and skin test sensitivity

Hunt and colleagues briefly reported from their controlled comparison of placebo, VIT and WBE that sIgE and sIgG levels measured at around 6 weeks after commencing treatment rose after VIT but not after placebo or WBE treatment. After one year of VIT, increased skin test sensitivity along with greater *in vitro* histamine release by leukocytes incubated with venom were also noted. Detailed data and statistical analyses were not provided.[245]

Rush VIT

In rush VIT, mean sIgE and sIgG levels begin to rise as early as 8 days, reaching a peak by 1-2 months.[267,276] Using an ultra-rush (6 hour) approach, one small study of vespid VIT demonstrated a rise in both sIgE and sIgG levels by 1 week, peaking by 2-4 weeks and declining gradually thereafter.[286] Another study of honeybee ultra-rush VIT observed an early rise in sIgG peaking at 9 months, and a transient elevation in sIgE at 2 months that was not statistically significant.[313] A reduction in skin test sensitivity may occur initially using such a regime,[314,315], which correlates well with decreased platelet reactivity to venom antigen (see 1.5.8.6 below).[315] However there are conflicting reports, with other investigators finding ultra-rush regime does not affect skin sensitivity to either venom or non-specific stimulation (codeine).[316] It may be that the rise in sIgE with ultra-rush VIT is relatively small, perhaps clouded by anti-idiotype IgG antibodies (see below) and skin tests may not be sufficiently sensitive to detect such changes.

Conventional VIT

The interpretation of studies comparing rush and conventional VIT are hampered by a lack of randomisation, with treatment allocation based on the season of presentation,[274] or using historical controls.[317] One study found that the mean rise in sIgE and sIgG reactivities appear to be the same by around the time a 100 ug maintenance dose is achieved irrespective of the schedule used, but did not report longer term data.[317] Another found that 3 months after initiating treatment patients receiving rush VIT tended to have higher sIgE and sIgG titres, and had a lower incidence of negative RAST and skin test results at one year compared to those receiving a conventional schedule. However, a seasonal allocation to treatment schedule (rush in spring/summer, conventional in

autumn/winter) was used rather than randomisation, and patients in the rush group of this study also tended to have higher RAST titres prior to receiving VIT.[274] Thus, irrespective of the schedule used, sIgE and sIgG antibody changes appear to follow a broadly similar pattern during the first few months. No published studies have utilised a diagnostic sting challenge immediately after the inpatient rush treatment, so whether rush VIT results in protection prior to the antibody response remains unknown.

Long term changes

Although increased sIgE and skin test sensitivity have been consistently demonstrated in the 3-12 months following the commencement of VIT whatever the initial schedule used,[245,246,269,277,318] sensitivity tends to fall gradually to pre-treatment levels within 1-2 years,[246,309,318] and a significant number of people loose their sIgE reactivity. The reported proportions of people on VIT with negative skin test and negative RAST at 3-5 years range from 10-20% for honeybee venom allergy,[274,282], and 27-54% for vespid venom allergy.[274,282,303] Declines in reactivity continue to occur after VIT has been stopped; in one long term study of 74 patients after a 5-year course of vespid VIT, 28% were skin test negative at cessation. 2-4 years later, 56-67% were skin test negative.[303]

One controlled (partly randomised) trial, in children with relatively mild reactions to wasp stings, has compared skin test sensitivity changes over a 1-year period between VIT and no treatment. The proportions demonstrating reduced sensitivity were 32/63 (44%) and 21/29 (72%) in the no treatment and treatment groups respectively ($p=0.08$ by the Fisher exact test). Some patients in both groups demonstrated increased sensitivity; again the difference between groups was not significant (13/63 with no treatment versus 2/29 with VIT, $p=0.11$).[256] In a comparison of honeybee VIT and WBE treatment, a small rise in sIgE was noted in the VIT group, but in both VIT and WBE groups sIgE had declined to below pre-treatment levels at the end of 1 year.[246] Thus, although the minority of people who develop negative skin tests and RAST during VIT may have long term protection from subsequent exposures (see 1.5.6 above), there is no convincing evidence that the declines in sIgE seen in these patients (and reported in many un-controlled studies) are in any way related to VIT.

Venom-specific IgG

Unlike sIgE, the levels of which are usually significantly elevated prior to VIT, sIgG levels are usually very low or undetectable prior to VIT.[269,276,286,319] The sIgG increases seen with VIT are sustained,[277,309,318] and remain significantly elevated for many years

after commencing VIT, when RAST and skin testing may indicate a loss of sIgE reactivity.[270,274,320] Overall however, sIgG levels are significantly higher in those who maintain their sIgE levels.[282]

Prior to immunotherapy, sIgG reactivity has been found more often in honeybee venom allergic than vespid allergic patients.[321,322] These baseline sIgG readings were attributed due to $sIgG_1$.[322] This finding was attributed to higher sting exposure rates in honeybee venom allergic compared to wasp venom allergic patients. During VIT both $sIgG_1$ and $sIgG_4$ subclasses increase,[319,322] however $sIgG_1$ levels fall rapidly to pre-treatment levels at the cessation of VIT whereas $sIgG_4$ levels are maintained for 3-5 years after VIT is stopped,[319,323] after which levels start to wane.[324] Protection appears to occur beyond the decline in sIgG.[300,320]

Passive immunization with hyper-immune serum can prevent reactions to sting challenge,[325] and can protect patients from reactions to VIT.[326] *In vitro* studies also indicate that the level of venom-specific IgG also correlates well with the ability of serum to inhibit antigen-mediated release from sensitised basophils.[327] Two groups studying honeybee VIT[305,328] and another of vespid VIT,[329,330] have found that patients reacting to sting challenge during the first 4 years of VIT had lower mean venom-specific IgG titres. It has been suggested that anti-IgE IgG antibodies may be the real "blocking antibodies" that prevent antigen-IgE interactions and FcεRI receptor cross-linking on the surfaces of effector cells,[331] and perhaps also lead to a reduction in sIgE and sIgG production.[332]

Failures of VIT to protect against sting reactions despite high levels of venom-specific IgG (including IgG_4) have been reported,[269,322,333] as well as a case where very high IgG titres were considered a possible cause of allergic symptoms.[274] Furthermore, antigen cross linking of cell membrane-bound IgG_4 has been demonstrated to activate basophils.[334] Prospective studies of patient reactions to honeybee and wasp stings prior to commencing VIT,[52] during maintenance VIT,[323] and years after stopping VIT,[237,335] have failed to find any correlation between venom-specific IgG levels and protection from systemic reaction to sting challenge. In one study, honeybee venom allergic patients having reactions tended to have higher venom-specific IgG than those that did not.[237,335]

1.5.8.3 Are we measuring sIgG, and does sIgE really fall with immunotherapy?

Venom-specific IgG is measured in various ways; radioimmunoassay,[245,269,303,321,329] RAST,[286,317] and sandwich ELISA.[169,274,319,322,323,335] All of these have in common an initial step whereby IgG binds to antigen, followed by a detection system that

identifies bound IgG. The existence of anti-IgE IgG antibodies has been known since as early as 1972,[336] and levels have shown to increase during venom immunotherapy in two studies,[331,337] but not in two other studies.[318,338] A combination of increasing sIgE and anti-IgE antibodies has the potential to give "false positive" results in venom-specific IgG assays. Anti-IgE may also effect sIgE assays by "hiding" sIgE from the assay and thus giving falsely low levels, an effect that may be reversed by heating to release bound IgE.[339-341] Anti-IgE IgG antibodies may vastly outnumber true antigen-specific IgG antibodies, and form circulating IgE-IgG complexes.[341] Although the effects of these mechanisms on serological tests are complex, they may explain many experimental results.

1.5.8.4 IgE-triggered mediator release in response to antigen

A 5-hour ultra rush schedule appears to reduce the degree of histamine release from leukocytes, as well as reducing skin test sensitivity.[314] Similarly, a 1-week rush vespid VIT has been found to reduce total blood histamine content and *in vitro* leukocyte mediator release (histamine and leukotrienes).[342-344] Normal mediator release patterns can be partially restored by the neutralisation of IL-10 and IFN-γ with monoclonal antibodies against these mediators, whereas prior to VIT the addition of exogenous IL-10 and IFN-γ reduces mediator release.[344] Consistent with the finding that cytokines can influence basophil and mast cell activation; previous *in vitro* studies had noted that venom-activated mononuclear cells from patients on maintenance VIT can modulate basophil histamine release.[345] Other basic research has suggested that deactivation of surface sIgE (perhaps by internalisation or shedding), after FcεRI receptor cross-linking due to antigen contact, may also contribute to the reduction in mediator release seen early after rush VIT schedule.[346]

Longer-term effects of immunotherapy, as well as the effect of more conventional outpatient approaches to immunotherapy on *in vitro* basophil mediator release are not well defined. Hunt et al. in their controlled trial of outpatient semi-rush VIT noted that at one year both skin test reactivity and basophil histamine release were increased. This same effect has also been noted during immunotherapy for pollinosis, with a gradual decline in histamine release over subsequent years.[347]

1.5.8.5 T-lymphocyte reactivity (proliferative and cytokine responses to antigen)

The proliferation of peripheral blood T-cells measured by radio labelled thymidine uptake in response to incubation with venom antigen varies according to methodology, VIT schedule, and timing of blood samples. Inpatient ultra-rush and rush VIT appears to be associated with a reduction in proliferative responses of leukocyte cultures.[313,348,349]

With a more conventional outpatient honeybee VIT schedule, CD-8 depleted leukocyte cultures show a trend towards an increased proliferative response at 4 weeks (although not statistically significant), with a significant reduction in proliferative response at 3 years.[350] A direct comparison of rush and conventional VIT schedules found that rush VIT causes an early reduction in proliferation at 1 week followed by an increase at 1 month with a gradual decline to below baseline at 1 year, whereas conventional VIT caused no change in proliferative responses until a decline occurring 1 year after commencing VIT.[348]

Increased IL-4:IFN-γ ratios have been shown to induce IgE and suppress IgG_4 production by peripheral blood mononuclear cells from allergic individuals, whereas a low ratio enhances IgG_4 production and suppresses IgE production.[351] Allergen-specific leukocytes, selected by culture in the presence of venom then re-stimulated, secrete increased amounts of IFN-γ and reduced amounts of IL-4 and IL-5 within 1-2 weeks of commencing ultra-rush VIT.[313,352] In a direct comparison of rush and conventional VIT, IFN-γ production were found to be elevated at 2 months, falling but remaining higher than baseline thereafter. IL-4 and IL-5 production fell initially with rush VIT, rising to pre-treatment levels at around 3 weeks then falling progressively thereafter. With conventional VIT, a progressive fall in IL-4 production, significant at 2 months and "undetectable" at 6 months has been noted.[348] This effect is sustained after 3 years of VIT.[350]

Flow cytometry analysis of peripheral blood mononuclear cells indicates that the proportion of IL-4 (TH-2 type) T cells within both $CD4^+$(T-helper) and $CD8^+$ (T-suppressor) groups falls by the end of a 7 day rush vespid VIT course, with a sustained reduction at 6 months. The percentage of IL-2/IFN-γ secreting (TH-1 type) does not seem to be altered by 7 days, but increases, again within both $CD4^+$(T-helper) and $CD8^+$ (T-suppressor) groups, by 6 months.[353] Associated with early cytokine changes in rush VIT, the yield of mononuclear cells from peripheral blood is reduced but rapidly recovers to normal levels; this affects all subgroups (monocytes, $CD4^+$ T cells, $CD8^+$ T cells, and NK cells).[348] An examination of blood counts reveals that at 7 days (immediately after completion of initial rush phase), leukocyte counts fall, with a fall in the % of lymphocytes but an increase in the % of neutrophils, returning to normal by 6 months.[353] Thus, sequestration of venom-reactive cells to peripheral sites may explain some *in vitro* changes seen early after rush VIT.

As well as confirming the early reduction in leukocyte proliferation and rapid shift from a TH-2 to TH-1 type response by peripheral leukocytes with rush VIT, Bellinghausen and colleagues demonstrated an increase in IL-10 secreting $CD4^+$ T cells. The addition

of anti-IL-10 antibodies restored proliferative responses, but did not restore IL-4 production and further increased IFN-γ production. The investigators postulated that IL-10 in this experimental model acted as a negative-feedback system to prevent an excessive TH-1 response.[349]

The mechanism by which the apparent switch from a TH-2 to TH-1 type response is induced by VIT remains unclear. Antigen-presenting cells may play a role (see 1.5.8.6 below). Histamine may impair such a switch, perhaps explaining the observation that H_1-blocker pre-medication, used to prevent reactions during VIT, may improve the long term efficacy of VIT (see 1.5.4 above).

Similarly, the mechanisms by which the TH-2 to TH-1 switch might cause hyposensitisation is unknown. Although there is a link between these types of T-helper cell activity and IgE and IgG production, these antibody effects do not appear to determine a successful clinical response (see 1.5.8.2 above). It may be that either or both of the TH-1/TH-2 and antibody changes during VIT are simply markers of another, as yet unidentified process.

1.5.8.6 Other cells and mediators

In vivo basophil activation (CD63 surface antigen expression) suggesting low-level stimulation and mediator release has been identified during rush vespid VIT.[354] Whether mediators so released are important in the mechanism of rush VIT remains unknown.

Platelet activation (cytotoxicity) in response to incubation with venom has been reported to be reduced immediately with rush VIT,[355] and corresponds closely with a reduction in skin test sensitivity (see 1.5.8.2 above).[315] Subsequent studies by the same group have found that IgG/IgE depleted serum from the VIT treated patients prevents the activation of platelets from untreated allergic subjects, an effect that was not antigen-specific.[356] The substance, named "platelet activity suppressive lymphokine", is secreted by $CD8^+$ T cells.[357]

An important issue to consider is the link between antigen administration during VIT and subsequent lymphocyte responses. Whether the mode of administration (subcutaneous with VIT versus intradermal with a sting) is important in this regard is unknown. Antigen presenting cells (APC) are considered a key link in the development of allergy, by exerting a TH-2 polarizing effect on lymphocytes,[358] so it is reasonable to postulate that they may have a key role in mediating the TH-1 polarizing effects of VIT. Antigen presented by dendritic cells to naive T cells in the context of MHC class

I with simultaneous secretion of IL-12 appears to suppress IgE production, and lento-viral transduction of dendritic cells to behave in this way in response to antigen has been achieved *in vitro*.[359] Tissue APCs, including dendritic cells, are difficult to study in a clinical setting. However, blood monocytes are circulating antigen-presenting cells that are more amenable to study. By day 15 after the commencement of rush VIT, purified blood monocytes show an increase in spontaneous release of IL-12 and TNF.[360]

Tolerogenic mechanisms may also come into play at the APC-effector cell level. Dendritic cells are known to express FcεRI receptors for IgE and are located close to tissue mast cells. As well as mediating some aspects of the inflammatory response to antigen, immunomodulatory cytokines are released and it has been suggested that post- FcεRI receptor events may be potential targets for immunotherapy in the future.[120] In the *in vitro* setting, low dose antigen presentation to T cells induces an IL-4 dominated response, whereas higher doses of antigen and a predominance of antigen presenting cells of the monocyte type facilitate a TH-1 type response.[361] Also, given the close apposition of APC and mast cells in skin, a direct inhibitory effect on mast cells also cannot be excluded.

1.5.8.7 Mechanisms of VIT- summary and concluding comments

A graphical summary of known changes in antibody levels, basophil mediator release and lymphocyte culture venom-induced responses (proliferation and cytokine release) during VIT is presented in Figure 1.7.

Early mechanisms of desensitisation, prominent during rush VIT, may involve mediator depletion and a circulating lymphokine that reduces effector cell reactivity. Changes in T cell response to antigen appear to be prominent during hyposensitisation and may be a key link. However, the events linked to changes in the IL-4:IFN-γ ratio (alterations in immunoglobulin levels) are not convincingly correlated to protection from sting challenge. Long-term reductions in sIgE levels may occur without VIT, and long term protection may occur in people with detectable sIgE and minimal sIgG. The issue of antibodies is further complicated by the analytical problems relating to auto-anti-IgE antibodies.

Just as there are multiple redundant arms to the anaphylactic response, so it can be postulated that there may be multiple redundant mechanisms by which the body becomes tolerant to antigens, and thus by which VIT has its effect. These changes probably differ according to the method and time elapsed since commencing

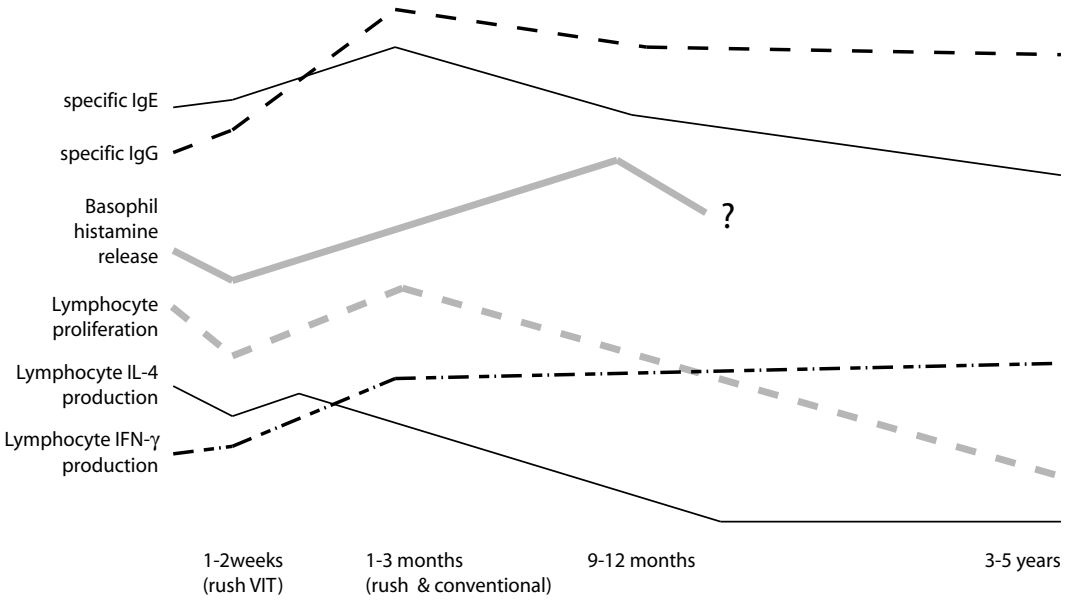


Figure 1.7: Summary of laboratory changes known to occur during VIT

immunotherapy. It is likely that many, if not all of the effects of VIT described above are simply markers of an underlying unknown mechanism.

1.5.9 New technologies and adjuvants

A number of strategies to enhance efficacy and improve the safety of specific (venom and aeroallergen) immunotherapy have been reported. These have included adsorption of allergens to *L*-Tyrosine,[362] adsorption to carbohydrate-based particles,[363] conjugation of allergen with cholera toxin subunit B,[364] conjugation of allergen with immunostimulatory oligodeoxynucleotide sequences,[365] co-administration with detoxified bacterial cell wall components such as monophosphoryl lipid A (MPL),[366] and immunotherapy using synthetic T-cell epitope peptides.[367] Some data has also suggested that the co-administration of IL-12 during immunotherapy may be useful.[368] To date these studies have been mainly performed in animals, using antibody measurements (augmented IgG and suppressed IgE responses) and lymphocyte culture cytokine responses to determine treatment efficacy. There have been two human studies. One tested T-cell epitope peptide immunotherapy for honeybee venom allergy on five humans with encouraging results.[367] The other enrolled 142 participants in a multi-centre randomised placebo-controlled trial of a tyrosine-adsorbed grass allergen extract co-administered with MPL, finding that 4 pre-seasonal injections per year were efficacious in producing sustained IgG responses and symptomatic relief.[366]

1.6 *Myrmecia* ant venoms

Myrmecia spp. venom is produced in venom glands (remarkable amongst the Hymenoptera for their slimness and considerable length) and stored in a venom reservoir (sac) (Figure 1.8). Studies of Australian ant venoms so far have used venom obtained by venom sac dissection. The larger species such as *M gulosa* may hold as much as 300 ug venom (dried weight) in their venom sacs, or 0.35% of body weight.[369] JJA venom sacs on average yield around 40 ug of venom each.[370]

1.6.1 Pharmacological properties

Pharmacological studies of *M gulosa* and *M pyriformis* venoms have identified histamine releasing, smooth muscle stimulating, hyaluronidase, phospholipase (A_2 and B), acid phosphatase, alkaline phosphatase, kinin-like, and haemolytic activities, an inhibitor of insect mitochondrial respiration, and histamine (~2% of dried venom weight).[371-374] JJA venom has been found to have very similar pharmacological activities to *M gulosa* and *M pyriformis* (including a histamine content of ~1% dried weight) but with slightly less phospholipase B, acid phosphatase, and alkaline phosphatase activities.[373] More recently, additional properties have been attributed to synthetic peptides derived from JJA venom. These are cytotoxic activity for pilosulin 1,[375] and hypotensive activity for pilosulin 2.[376]

1.6.2 Allergenic components

Early studies using paper electrophoresis separated *M gulosa* venom into 7 bands,[369] and starch gel electrophoresis has been reported to separate *M pyriformis* venom also into 7 bands.[377] More recently, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) separation has been able to identify 16 bands in JJA, *M tarsata*, and *M pyriformis* venoms, and 14, 13, and 12 bands in *M nigrocincta*, *M simillima* and *M gulosa* venoms respectively.[27] These ranged in estimated molecular weight from 2-94 kDA; the higher molecular weight (>30 kDa) were well resolved but only lightly stained. IgE-binding components all had estimated molecular weights ranging from 2 to 25 kDa (Figure 1.9). All JJA venom components that bound IgE were <8.5 kDa. The light staining and lack of IgE recognition of higher molecular weight bands might be an artefact of venom sac dissection. Honeybee and wasp venoms obtained in this way have been shown to be contaminated by higher molecular weight abdominal tissue proteins.[378,379]

JJA-derived cDNA encoding two major allergens recognised by IgE from allergic patients have been identified and named *Myr p 1* and *Myr p 2*, encoding peptides of 112 and 75 amino acid residues respectively.[380-383] These encode precursor peptides

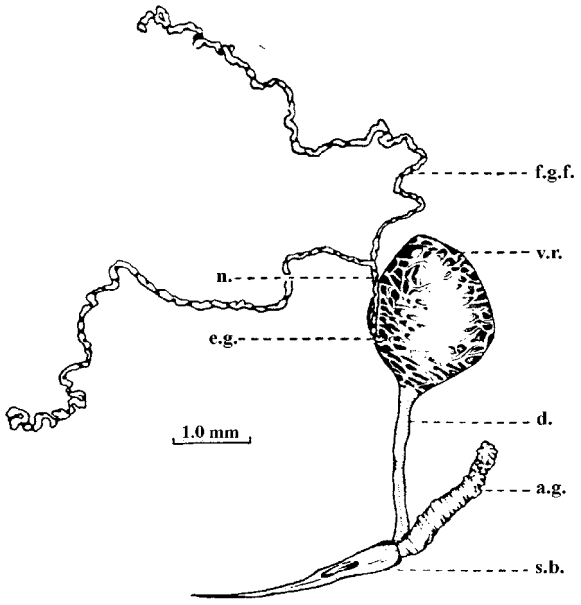


Figure 1.8. Components of the venom apparatus of the bull ant, *Myrmecia gulosa*. Abbreviations: *a.g.*, accessory (Dufour's) gland; *d.,* venom duct; *e.g.*, point of entry of free venom gland between outer and inner layers of reservoir wall; *f.g.f*, free gland filaments; *n.*, "neck" region of venom gland; s.b., sting bulb; *v.r.*, venom reservoir. Reproduced, with permission from: Cavill GW, Robertson PL, Whitfield FB. Venom and venom apparatus of the Bull Ant, Myrmecia gulosa (Fabr.). Science 1964;146:79-80. Copyright 1964 American Association for the Advancement of Science.

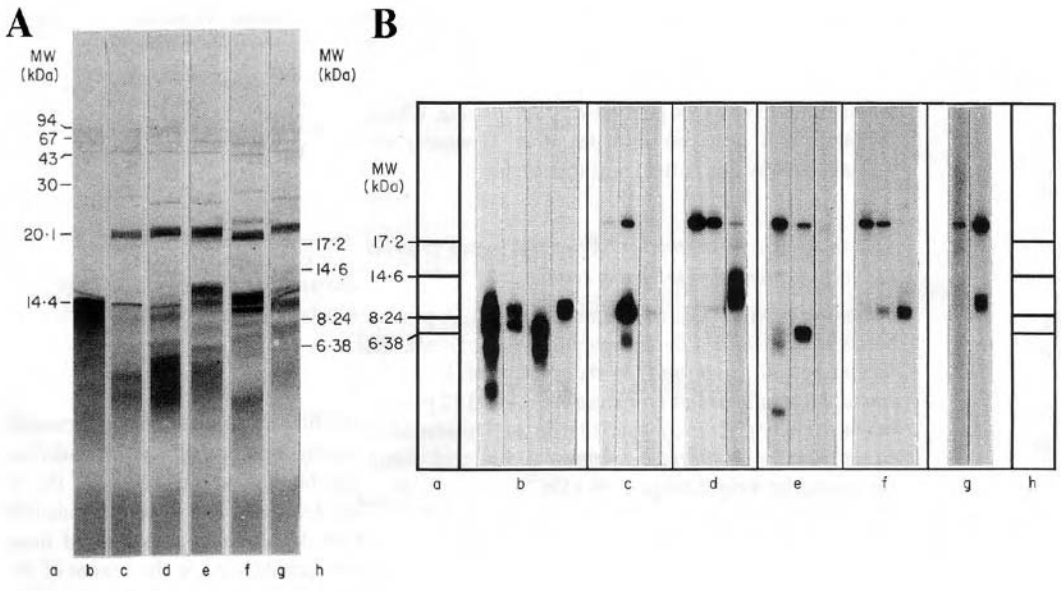


Figure 1.9. SDS-PAGE and immunoblotting of six *Myrmecia* venoms. (A) Amido black staining of nitrocellulose transfer. (B) Immunoblots (using individual patient sera) of IgE-binding components. (a) and (h) molecular weight markers; (b) JJA (*Myrmecia pilosula*); (c) *Myrmecia nigrocincta*; (d) *Myrmecia tarsata*; (e) *Myrmecia pyriformis*; (f) *Myrmecia simillima*; (g) *Myrmecia gulosa*. Reproduced, with permission from: Street, M.D., et al., Immediate allergic reactions to Myrmecia ant stings: immunochemical analysis of *Myrmecia* venoms. Clin Exp Allergy, 1994. 24(6): p. 590-7. Copyright 1994 Blackwell Publishing Ltd.

A : *Myr p 1*

				5					10					15					20
Met	Lys	Leu	Ser	Cys	Leu	Leu	Leu	Thr	Leu	Ala	Ile	Ile	Phe	Val	Leu	Thr	Ile	Val	His
				25					30					35					40
Ala	Pro	Asn	Val	Glu	Ala	Lys	Asp	Leu	Ala	Asp	Pro	Glu	Ser	Glu	Ala	Val	Gly	Phe	Ala
				45					50					55					60
Asp	Ala	Phe	Gly	Glu	Ala	Asp	Ala	Val	Gly	Glu	Ala	Asp	Pro	Asn	Ala	**Gly**	**Leu**	**Gly**	**Ser**
				65					70					75					
Val	**Phe**	**Gly**	**Arg**	**Leu**	**Ala**	**Arg**	**Ile**	**Leu**	**Gly**	**Arg**	**Val**	**Ile**	**Pro**	**Lys**	**Val**	**Ala**	**Lys**	**Lys**	**Leu**
				85					90					95					100
Gly	**Pro**	**Lys**	**Val**	**Ala**	**Lys**	**Val**	**Leu**	**Pro**	**Lys**	**Val**	**Met**	**Lys**	**Glu**	**Ala**	**Ile**	**Pro**	**Met**	**Ala**	**Val**
				105					110										
Glu	**Met**	**Ala**	**Lys**	**Ser**	**Gln**	**Glu**	**Glu**	**Gln**	**Gln**	**Pro**	**Gln**								

B : *Myr p 2*

				5					10					15					20
Met	Lys	Leu	Ser	Cys	Leu	Leu	Leu	Thr	Leu	Ala	Ile	Ile	Phe	Val	Leu	Thr	Ile	Val	His
				25					30					35					40
Ala	Pro	Asn	Val	Glu	Ala	Lys	Ala	Leu	Ala	Asp	Pro	Glu	Ser	Asp	Ala	Val	Gly	Phe	Ala
				45					50					55					60
Asp	Ala	Val	Gly	Glu	Ala	Asp	Pro	**Ile**	**Asp**	**Trp**	**Lys**	**Lys**	**Val**	**Asp**	**Trp**	**Lys**	**Lys**	**Val**	**Ser**
				65					70					75					
Lys	**Lys**	**Thr**	**Cys**	**Lys**	**Val**	**Met**	**Leu**	**Lys**	**Ala**	**Cys**	**Lys**	**Phe**	**Leu**	**Gly**					

C: Proposed sub sequences

	Isotopic Average Mol Wt
Myr p 1 57 → 112 (pilosulin 1)	6052
Myr p 2 49 → 75 (pilosulin 2)	3212
Myr p 1 68 → 112	4938
Myr p 1 65 → 112	5279
Myr p 1 71 →?112	4655
Myr p 1 86 → 112	3069

(Myr p 1 68 → 112 possibly as 2 isomers)

Figure 1.10: A & B: Published *Myr p 1* and *Myr p 2* (cDNA-derived) sequences.[380-383] **C:** Sub sequences proposed to be expressed in native *M pilosula* venom; sequences thought to represent the major IgE binding bands on SDS PAGE are in bold.[384]

that have a common leader sequence, differing by only three amino acids in the first 47 residues. However, both appear to undergo extensive post-translational modification and using SDS-PAGE, immunoblotting and 8 cycles of N-terminal amino acid sequencing, it has been proposed that whole venom contains six sub sequences of *Myr p 1*.[384] These primary cDNA-derived peptide sequences and proposed sub sequences are summarised in Figure 1.10.

IgE binding to venom separated by SDS-PAGE at the ~7.5kDa band has been attributed to a *Myr p 1* derived peptide (residues 57→112),[384] whereas binding at the ~8.5 kDa and 2-4kDa bands has been attributed to *Myr p 2* derived peptides.[383] These major sub sequences, apparently the predominant forms expressed in native venom, have been referred to as "pilosulin 1" and "pilosulin 2" respectively.[375,376] The apparent recognition of pilosulin 2 in two separate SDS-PAGE bands is thought to be due to the presence of multimeric forms of pilosulin 2 within whole venom. Given that pilosulin 2 contains 2 cysteine residues, the 8.5kDa peptide has been proposed to contain pilosulin 2 in a disulfide linked complex - either with itself or with another non-allergenic peptide(s).[384]

1.7 Literature review summary

Although there are many hundreds of species of stinging ants in Australia, according to serological and clinical data JJA stings appear to be responsible for the majority (perhaps >90%) of ant venom anaphylaxis in this country, particularly in southeastern Australia where *Myrmecia* ants are most commonly encountered. Data from country Victoria suggests that in areas where these ants are common, the prevalence of JJA venom allergy in the adult population may be in the order of 2.9%. Deaths recognised as being due to *Myrmecia* sting anaphylaxis appear to have been largely confined to the island of Tasmania, and due mainly to JJA stings.

Insect sting anaphylaxis appears to be typical of anaphylaxis in general except that delayed phase reactions may be less prominent, and hypotensive (shock reactions) appear to be more common than asthma-type reactions when compared to food anaphylaxis. A number of grading systems are available for use in clinical research; although various adaptations of the Mueller grades are used most often, there is little data available to validate any system. Our understanding of the pathophysiology and treatment of anaphylaxis is based largely on animal studies, retrospective human case series, and a small number of prospective observational human studies. Vasodilation, fluid extravasation and bronchospasm appear to be the major pathophysiological

components of anaphylaxis, for which volume resuscitation and adrenaline are appropriate treatment by providing "physiological antagonism" of the anaphylactic process. However, a direct effect of anaphylactic mediators on cardiac and central nervous system tissues may be significant in some reactions.

The natural history of insect sting allergy is unpredictable. A tendency for the majority of people with a history of sting anaphylaxis to loose their clinical sensitivity, as well as a natural fluctuation in reaction severity over time, has previously contributed to erroneous confidence in ineffective hyposensitisation treatments. Age and prior reaction severity appear to be the only factors that determine subsequent reaction occurrence and severity. Currently available diagnostic tests are able to detect either the presence of sIgE or the cellular processes that result from activation of surface-bound sIgE. No *in vitro* test appears to have any advantage over VST. Although VST may be helpful to confirm the likely causative insect of a prior reaction, the specificity of this test for indicating subsequent clinical reactivity is poor. Even the diagnostic sensitivity of skin testing in detecting those at risk has been questioned in the setting of vespid allergy.

20 years of clinical experience and two controlled (but not randomised or double-blinded) trials indicate that VIT is an effective treatment for reducing reaction risk in people with honeybee and wasp sting allergy. However, a critical review of available studies reveals a number of methodological shortcomings and by current standards, the evidence for efficacy is suboptimal. The mechanisms of VIT are unknown and so far, no laboratory markers appear to define successful treatment.

The allergenic components of JJA venom and patient sera reactivity have been studied in detail, laying a foundation for further study to determine the efficacy of JJA VIT.

Chapter 2: Research objectives and outline

2.1 Objectives

The main aim of this research was to develop a venom immunotherapy for JJA allergy. This required the collection of data to facilitate planning for service provision, knowledge of the natural history of JJA allergy and predictors of reaction severity to guide the application of VIT, and finally a trial to determine the efficacy and tolerability of VIT. Therefore, a number of major objectives were identified:

i. To determine the prevalence, severity and natural history of JJA venom allergy in the Tasmanian population;

ii. To evaluate clinical predictors of accidental sting reaction occurrence and severity in people with known JJA venom allergy;

iii. To assess a set of available *in vitro* laboratory tests for their diagnostic utility to predict clinical sensitivity as determined by a deliberate sting challenge, and;

iv. To test the tolerability and efficacy of outpatient VIT for preventing JJA sting anaphylaxis.

The trial structure also provided an opportunity to examine the clinical and biochemical features of JJA sting anaphylaxis induced in a controlled environment, including an assessment of a resuscitation protocol, and to identify potential *in vitro* markers of successful VIT.

2.2 Research outline

Section II of this thesis reports the epidemiology of JJA sting anaphylaxis in Tasmania, starting with a review of deaths occurring in the southern region of Tasmania serviced by the Royal Hobart Hospitals' forensic service. A retrospective analysis of ED presentations with sting anaphylaxis over a 9-year period was performed and compared with population sting exposure rates and prevalences of systemic allergy to common local stinging insects. Clinical and serological characteristics of a group of JJA allergic volunteers were then assessed and these people were followed up over a 4-year period

to identify predictors of subsequent reaction severity to accidental stings that might facilitate the selection of people most likely to benefit from VIT.

Section III describes a randomised, double-blind, placebo-controlled crossover clinical trial of JJA VIT. As well as examining the efficacy and tolerability of VIT, this includes a detailed description of reactions to sting challenge including clinical features, response to a treatment protocol using intravenous adrenaline and fluid resuscitation, and an assessment of the diagnostic utility of serum mast cell tryptase and plasma histamine for the diagnosis of anaphylaxis. The section concludes with an analysis of the ability of laboratory tests (sIgE analysis and in-vitro venom-induced basophil degranulation and mediator release, leukocyte proliferation and IL-4 production), and venom skin testing to predict the outcome of deliberate sting challenges in the placebo group of the clinical trial, and examines the changes in these laboratory parameters during VIT.

2.3 Thesis structure

Structured discussions have been used (statement of principal findings; study strengths and weaknesses; comparison with related studies; interpretation; unanswered questions and future research),[385] and for the main clinical trial the CONSORT checklist (2001 revision) for reporting clinical trials has been used.[386]

PART II: EPIDEMIOLOGY OF JACK JUMPER ANT STING ALLERGY IN TASMANIA

Chapter 3: Deaths

3.1 Introduction

It is likely that JJA sting deaths follow the same patterns observed for other stinging insects. However, prior to undertaking and publishing the following work, no JJA sting anaphylaxis deaths had been reported with any detail in the medical literature. The objective of this section was to describe the clinical and serological features of JJA sting deaths occurring in southern Tasmania. Ethical approval was granted by the Royal Hobart Hospital Ethics Committee.

3.2 Methods

A manual search of the Royal Hobart Hospital forensic register from January 1980 to December 1999 inclusive was performed to identify deaths attributed to JJA sting. This register recorded the final diagnoses from all post mortem studies performed in the hospital, which provided the only service for post-mortem investigations in the southern region of Tasmania, for a stable population of 229,000 during the period studied (Australian Bureau of Statistics, 1996). Clinical notes including police records, ambulance report forms, hospital and forensic notes were reviewed.

From 1994 onwards as part of the forensic investigations, sera were sent to the Kolling Institute Molecular Immunology Unit (Dr Brian Baldo and Qi Xuan Wu), Royal North Shore Hospital Sydney, for immunological studies. Serum mast cell tryptase was determined by radioimmunoassay.[387,388] During the time span of this study, methodologies for tryptase measurement changed from the measurement of mature tryptase (Pharmacia RIA, reference range <2 ug/L) to the measurement of both mature and precursor forms of tryptase (Pharmacia UniCAP, reference range <12 ug/L), so reference intervals differed between cases. Specific IgE reactivities were determined by RAST, with radioactive tracer uptakes of >2% considered positive.[27,381] Inhibition studies were also performed on the most recent case. In these studies, serum samples were pre-incubated with venom or peptide allergens (*Myr p 1*, *Myr p 2* and another previously undescribed synthetic peptide, *Myr p 3*) prior to analysis by RAST. Tubes containing serum without pre-incubation were used as a baseline to calculate percentage inhibition by the pre-incubation venom or peptide. *Myr p 3* had been

synthesised to represent a heterodimer thought to be the main form in which *Myr p 2* is expressed in JJA venom.[389]

3.3 Results

Four deaths were identified, giving a mortality rate due to ant stings in southern Tasmania during the 20 year study period of 0.9 per million per annum. All deaths occurred during the last 10 years of the study. Post-mortem serum mast cell tryptase was substantially elevated in only one of three cases where this was measured. IgE recognition of JJA venom was evident in all three sera that were tested, with one showing strong cross-reactivity to all *Myrmecia* venoms tested. Interestingly, in the last case where detailed serological studies were performed, synthetic peptides did not appear to bind venom-specific IgE.

All deaths occurred in males aged over 40 years, who had various comorbidities (cardiac disease in three cases), or used medications (ACE inhibitors and/or beta blockers in two cases, promethazine injection combined with alcohol intoxication, benzodiazepine and obstructive sleep apnoea in another case) that may have contributed to death. Three of the four had a past history of allergic reactions to *Myrmecia* stings. None received adrenaline prior to the onset of cardiac arrest. One believed he was protected by prior treatment with jumper ant whole body extract.

The following are summaries of each case:

Case 1

In 1989, a 49 yr old man working in bush stated that a JJA had stung him. Because of known allergy to these stings he took two antihistamine tablets. He was left alone for 15-20 minutes and when his companions returned he was dead.

Past history included hypertension treated with enalapril. Post mortem revealed cardiomegaly, acute on chronic pulmonary congestion and "unusually fluid blood". The pathologist discounted ant sting anaphylaxis as "exceedingly rare". Tissue from a suspected forearm bite was negative for snake venoms (Dr Struan Sutherland, Commonwealth Serum Laboratories). Tests for serum tryptase and insect venom-specific IgE were not available.

Case 2

In 1995, a 62 yr old man was thought to have been stung by a JJA whilst fishing, the ant being seen on his arm by a companion. Tongue and lip swelling and breathlessness developed

shortly thereafter. When a paramedic arrived 20-30 minutes later he was in cardiac arrest. Resuscitation was unsuccessful.

Past history and allergies were not recorded. Post mortem revealed severe oedema of lips and tongue, oedema of the upper airways, extreme lung congestion, diffuse severe atherosclerosis with occlusions of 50-75% and marked hypertensive left ventricular hypertrophy. Toxicological screening revealed a blood alcohol of 0.207 g/100 ml with no other drugs or substances identified. A tryptase level from blood taken 3 days after death was 2.73 ug/L (reference range <2.0). IgE specific to JJA venom was present (uptake 15.2%). Reactivity to other venoms was not tested.

Case 3

In 1995, a 40 yr old man reported to his friends that he had been stung by a "bull ant" (a term commonly used by locals, including some medical practitioners, when referring to M pilosula*). He injected himself with promethazine 50 mg and continued to drink alcohol. Some time after this he was noted to be slurring his words, falling over on his way to the bedroom. The precise timeframes for these events are difficult to determine from the available records. He was found deceased in bed in the early hours of the morning.*

Past history included obstructive sleep apnoea and progressively worsening systemic allergic reactions to "bull ants" for which he was prescribed intramuscular antihistamine and an adrenaline puffer. The available medical records make no mention as to whether the term "bull ants" was in reference to JJA or to M forficata*. He was known to be a heavy alcohol user and was also prescribed diazepam and fluoxetine.*

Post mortem was unremarkable apart from a fatty liver. Toxicological screening revealed a blood alcohol of 0.218 g/100 ml and non-toxic concentrations of diazepam and fluoxetine. A tryptase level from blood taken three days after death was 2.72 ug/L (reference range <2.0). Testing for venom specific IgE revealed high uptakes for JJA (31.7%), and other Myrmecia *species tested (*M nigrocincta *24.5%,* M tarsata *42.1%,* M pyriformis *39.6%,* M simillima *39.8% and* M gulosa *37.0%).*

Case 4

In 1999 while working in his backyard in Hobart, a 65 year old man complained that he had just been stung on the knee by a JJA. He immediately felt unwell, itchy and short of breath. An ambulance was called and he collapsed.

A paramedic crew arrived 5 minutes later when he was found to be deeply cyanosed with no palpable pulses. He had a generalised urticarial rash and was making an occasional respiratory effort without any movement of air. CPR was commenced and intravenous adrenaline administered. ECG demonstrated idioventricular rhythm. Bag-valve-mask ventilation was ineffective and gastric insufflation with regurgitation of stomach contents was observed. Paramedics administered a total of 13 mg of adrenaline and 1 litre of crystalloid

intravenously. At no stage was a pulse detected. On arrival in hospital 50 minutes after the arrest, laryngoscopy revealed marked laryngeal oedema and a gum-elastic bougie was required to achieve intubation. Resuscitation attempts were ceased shortly thereafter.

Past history included allergy to JJA, with unconsciousness on several occasions. Fifteen years prior to his death, hyposensitisation was attempted with crushed jumper ant whole body extract (Commonwealth Serum Laboratories, Melbourne). Since then he had been stung once, with what the family recalled to be a less severe reaction, leading him to believe that he was protected from further stings. He also had a history of atrioseptal defect repair, chronic atrial fibrillation and impaired left ventricular function. Medications at time of presentation included warfarin, digoxin, bumetanide, carvedilol and fosinopril.

Post mortem revealed considerable oedema of the larynx, aryepiglottic folds and adjacent pharyngeal tissues (Figure 3.1). The lower airways were clear, indicating that aspiration had not occurred. There was no significant coronary artery disease, however there was marked dilation of the tricuspid and mitral valves and all cardiac chambers.

Serum tryptase from blood taken 4h after termination of resuscitation was 51.6 ug/l (reference range <12). Significant IgE binding was seen with the JJA and honeybee venoms, with uptakes of 10.1% and 2.7% respectively. Uptakes to three synthetic venom peptides Myr p1-3 were 0.4%, 3.2% and 1.0% respectively. No significant IgE reactivity with European wasp or other Myrmecia *venoms was detected. Inhibition studies (Figure 3.2) found whole JJA venom to be a potent inhibitor of IgE binding to JJA venom in the solid phase. Of the synthetic peptides, only Myr p 2 showed significant inhibition. However, this was markedly less than for whole venom; although 80-90% inhibition of IgE binding was achieved with whole venom, synthetic peptides achieved only 35% inhibition, and only at much higher concentrations. Honeybee venom did not show a significant inhibitory effect on IgE binding to JJA venom (Figure 3.3).*

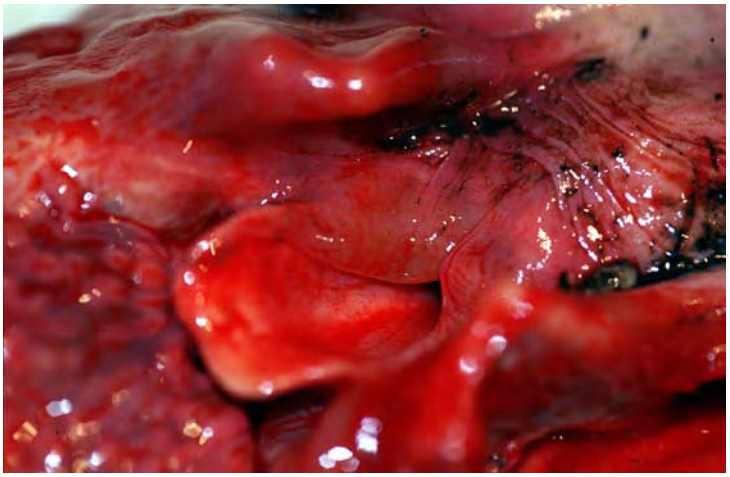


Figure 3.1: Post mortem appearance of the larynx in case 4; note oedema of aryepiglottic folds and adjacent pharyngeal tissues. Photograph courtesy of Dr G Robert H Kelsall, Director of Forensic Pathology, Royal Hobart Hospital.

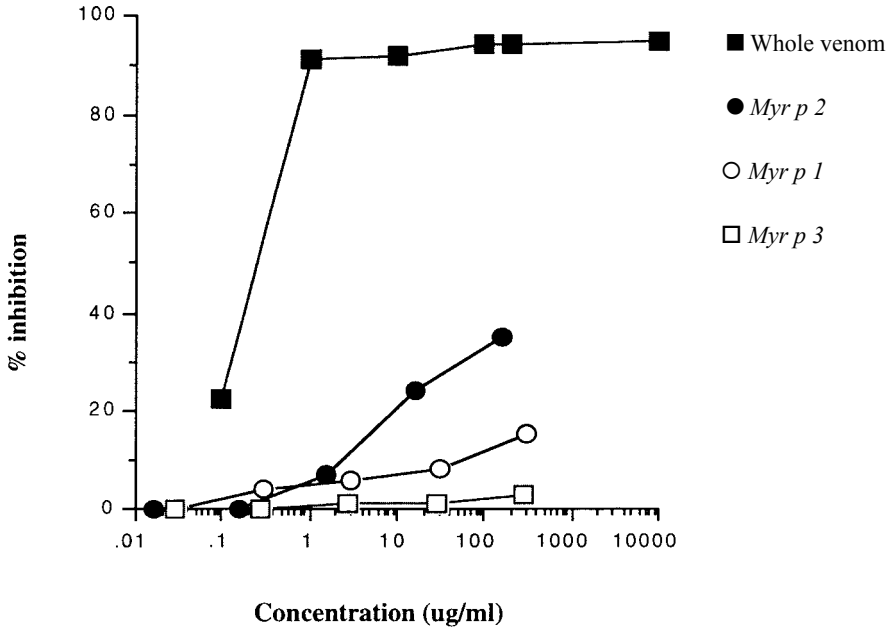


Figure 3.2: Inhibition of IgE binding to whole venom in the solid phase by whole venom and synthetic venom peptides

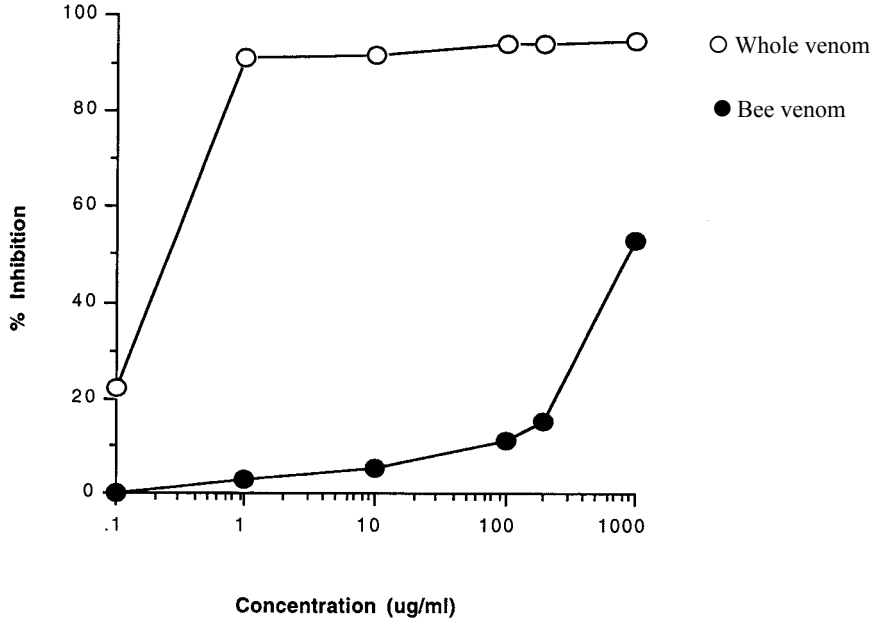


Figure 3.3: Inhibition of IgE binding to whole venom in the solid phase by bee venom

3.4 Discussion

3.4.1 Principal findings

All four deaths occurred in adult males with significant cardiac comorbidities &/or taking medications that may have contributed to death. Three had sought medical care for ant sting allergy previously. Detailed analysis of the sera from one death suggests that currently available synthetic peptide allergens may not completely reproduce the immunological activity of native JJA venom. IgE cross-reactivity between closely-related *Myrmecia* species was identified in one case; that this represented true cross-reactivity rather than individual sensitisations is almost certain; the geographical distribution of these species means that exposure to all is exceptionally unlikely.

3.4.2 Study strengths & weaknesses

The small numbers of deaths identified prevented any firm conclusions being made about death rate or factors predisposing to death. That all deaths were confined to the last 10 years of the study (with the first case initially dismissed because the pathologist considered death due to JJA anaphylaxis to be "exceptionally rare") suggests under-recognition has been the norm. Dr Paul Clarke brought the potential extent of severe JJA sting allergy to the attention of the Tasmanian medical community in 1986,[2] after which all of these deaths were diagnosed. No attempt was made to review all suspicious deaths during the study period.

Only two deaths were directly observed by trained personnel (cases 2 and 4). Case 1 seems highly likely to be a sting anaphylaxis death but case 3 must be regarded as suspicious; unfortunately, it cannot be certain that *M pilosula* was the causative insect rather than *M forficata*. Furthermore, the mode of death remains uncertain.

3.4.3 Comparison with related studies

Three of the four victims in this study had a known sting allergy for which they had sought medical attention. In contrast, analysis of the available mortality studies indicates that overall only 14% of victims are identified as having a prior history of systemic sting allergy (see 1.2.4.2, Table 1.4). This supports the assertion made above that only a proportion of JJA sting deaths were identified, diagnosed because of prior history, with other cases going unrecognised in the absence of such history to alert the investigating pathologist. In approximately one half of deaths caused by anaphylaxis, no cause of death may be evident at post mortem which may also contribute to under-reporting.[156] Similarly, only two of this series of four deaths had diagnostic features of anaphylaxis at post mortem.

The finding of markedly elevated tryptase levels in only one case (with only marginal elevations in the other two that were tested) is consistent with current knowledge of the behaviour of this marker during anaphylaxis and after death. Tryptase half-life is 2 hours with an intact circulation, but increases to 4 days after death.[390] Tryptase levels do not increase after death.[391] One study, including only 7 anaphylactic deaths, found post mortem tryptase to be 86% sensitive and 88% specific using 10 ug/L as a cut-off.[392] However, tryptase is not elevated in many acute allergic reactions despite elevated histamine levels,[134] and may not enter the circulation until 30 minutes after exposure, peaking 1-2 hours following a sting if the circulation remains intact.[390] In sting deaths, although there may be delays of several hours from sting until severe compromise occurs, the median time to cardiac arrest is only 15 minutes.[67,99] Therefore, as well as lacking perfect sensitivity in people who do not die, where death rapidly supervenes tryptase levels may not have the opportunity to rise. Furthermore, the level of tryptase rise is related to the degree of hypotension,[131] so a prominent elevation may not be evident in patients dying from airway obstruction and hypoxia.

The absence from this study of deaths in young healthy individuals repeats the pattern observed in much larger studies of deaths due to honeybee and wasp sting allergy (see 1.2.4.2 above, Table 1.4), and is consistent with findings by van der Linden's group that older people are more likely to experience hypotensive reactions.[52] The observation that all four deaths were associated with significant comorbidities is also consistent with some, but not all, previous studies (see 1.2.4.2 above, Table 1.4).

The regular use of angiotensin-converting enzyme (ACE) inhibitors in two cases, combined with a b-blocker in one case, may have contributed towards death or may simply have been a marker of underlying conditions that determined outcome. ACE (kininase) inhibitors can trigger severe anaphylaxis in patients undergoing venom immunotherapy,[169] possibly due to decreased breakdown of vasodilator kinins activated during anaphylaxis &/or compromise of compensatory renin-angiotensin system activation.[40] Beta-blockers may impair both the endogenous adrenergic stress response and counteract and imbalance the effect of exogenous adrenaline. Interestingly, reduced renin-angiotensin system activity despite normal kininase activity is seen in patients with severe venom allergy, compared to healthy controls.[393] Drugs that produce selective inhibition of the angiotensin II-1 (AT_1) receptor, such as losartan, may have less deleterious effects. With haemodialysis, losartan may be associated with a lower incidence of anaphylactoid reactions than kininase inhibitors,[394] however no data is available with regard to anaphylactic reactions to external allergens.

Since the findings from this section were first reported,[395] a national ant sting mortality study has been published.[162] Only two additional cases were found during the same 20 year study period; one of these was from northern Tasmania and attributed to JJA and the other was from New South Wales, probably due to *M gulosa* or *M pyriformis*. In the later case, antibody cross-reactivity between gulosa group species was again evident, a pattern that has been noted in previous serological studies of ant venom allergic individuals.[27]

Following on from the serological findings in case 4, an analysis of IgE binding against *Myr p 1*, *Myr p 2* and *Myr p 3* synthetic antigens using sera from 300 JJA sting allergic patients has been carried out. This study reported that 20% of sera recognising whole venom did not recognise any of the synthetic peptides. Several of the sera not recognising synthetic peptides were examined for IgE binding activity against SDS-PAGE separated whole venom. A number of high molecular weight IgE-binding bands were identified at ~11.7, ~16.9 and ~43.5 kDa.[389]

3.4.4 Interpretation

JJA sting deaths follow the pattern seen with other sting allergies in that deaths appear to occur mainly in people over the age of 30. Co morbidities and medications may have an important contribution towards death in some cases.

In southern Tasmania where JJA are common and medical awareness of the problem is high, the rate of recognised mortalities appears to be high. In comparison, national figures are likely to be inaccurate, particularly for rural areas where JJA stings are frequently encountered, but death certificates are usually provided by local practitioners with little (if any) investigation.

This case series highlights some additional points with regard to sting allergy. Firstly, a number of people who die are aware of their allergy and there is potential to prevent deaths with an effective immunotherapy. It is also reasonable to assume that some deaths might be averted by the prescription of adrenaline for self-injection (although the efficacy of this treatment in very severe reactions is by no means proven). One victim believed he was protected by whole crushed body extract immunotherapy, and the occurrence of a less severe reaction after that treatment appears to have further misled him. This emphasises the need for careful patient education, and for any new preventative immunotherapy to be rigorously proven by a well-designed clinical trial.

Currently available synthetic peptides may not account for the total allergenic activity of JJA venom as represented by IgE binding. The higher molecular weight IgE binding

bands identified in JJA venom may represent either previously unrecognised venom allergens, abdominal protein contamination &/or aggregates of smaller peptides. This uncertainty has significant implications for immunotherapy; without further studies, it cannot be assumed that the currently available synthetic peptides will be effective substitutes for native venom immunotherapy.

3.4.5 Unanswered questions

The contribution of co morbidities and regular medications towards insect sting reaction severity remains uncertain. The true extent of ant sting mortality in Australia remains to be defined. Further studies into the IgE binding components of JJA venom are required to facilitate the standardisation of venom extracts &/or synthetic analogues for immunotherapy.

Chapter 4: Population prevalence and emergency department presentations

4.1 Introduction

The importance of JJA stings as a cause of anaphylaxis in Tasmania has been recognised since the late 1980's.[2] Subsequent clinical and serological data has suggested that the majority of ant venom allergy in southeastern Australia may be due to the JJA.[27,28] However, researchers have not previously attempted to determine JJA allergy prevalence in a whole population (including children), or to identify factors associated with severe reactions. Knowledge of these is required to enable rational planning for the provision of venom immunotherapy.

In addition to JJA, anecdotally recognised causes of sting anaphylaxis in Tasmania include *Apis mellifera* (honeybee) and *Vespula germanica* (European wasp). Another *Myrmecia* ant known to some locals, although not generally recognised as a cause of anaphylaxis, is the larger (20 mm) "Inchman". Our field trips around Tasmania have revealed this ant to be represented by one species, identified as *Myrmecia forficata* by CSIRO Entomology, Canberra. Another *Myrmecia* thought to be endemic to Tasmania is *Myrmecia esuriens*, but this is only very rarely encountered. Bumblebees (*Bombus* spp) have recently become established (becoming more widespread and frequently encountered since this research was begun), but because of their passive nature only very rarely sting humans. A solitary native flower wasp known as the "blue ant" or "bluebottle" (*Diamma bicolor*) is also encountered occasionally.

This section aimed to determine the population prevalence of JJA sting allergy in Tasmania, to compare these findings with emergency department presentations with sting anaphylaxis, and to identify patient characteristics associated with severe reactions. The Royal Hobart Hospital Ethics Committee granted ethical approval.

4.2 Methods

4.2.1 Emergency Department presentations

Royal Hobart Hospital ED presentations with sting anaphylaxis for an 8-year period to September 1998 were identified from a computerised tracking system and the clinical notes (including referral letters and ambulance report forms) obtained. Data on reaction severity, treatment with adrenaline, and likely causation as assessed by the

treating doctor were collected. Only basic demographics (age and sex) and treatments administered were analysed from this part of the study, due to uncertainty regarding the accuracy of other data (past history, regular medications etc) in the medical records.

The severity grading system used (Table 4.1) was based on a system developed by U. R. Müller,[11] which was itself a major modification of a system originally proposed by H. Mueller.[36] This system was chosen for the differential weighting of objective versus subjective indicators of reaction severity for grades II and III. A further modification was made for this research so that the most severe grade (IV) was according to major sting challenge studies, where a simplified definition of severe reactions identifying symptoms and signs indicating hypotension, applied retrospectively, appeared to be a sensitive tool for detecting people with severe allergy who would have a hypotensive reaction to subsequent deliberate sting challenge.[52,198]

4.2.2 Population sting exposure and allergy prevalence

In 1998, households were randomly selected from Tasmanian telephone directories with the aim of securing data from 1000 subjects. Data was collected for all members of the household irrespective of age. Structured telephone interviews by a market research company with medical research experience (Myriad Consultancy Market Research, Bellerive, Tasmania) collected demographic and sting exposure data for the best-known local insects (jack jumper, honeybee and wasp).

Telephone interviewers were required to confirm a standardised JJA description with people who stated that they had been stung by this insect. It was assumed that the general population was familiar with the appearance of honeybees and European wasps. People reporting allergic symptoms following a sting were referred to the clinical research team for further assessment. If subjects consented and were able to provide serum samples, these were sent to the Kolling Institute Molecular Immunology Unit, Royal North Shore Hospital Sydney (Dr Brian Baldo, Qi Xuan Wu), for venom-specific IgE analysis by RAST.[27,381] Results were expressed as percentage uptake of anti-IgE to serum IgE bound to venom on a solid phase.

4.2.3 Statistical analysis

For the random population survey, proportions and confidence intervals were determined by the Binomial test. For the analysis of reaction severity, dichotomous outcome variables were defined by reaction occurrence, occurrence of a grade III-IV (severe) reaction, or occurrence of a grade IV (hypotensive) reaction. Associations between each of these outcomes and each baseline characteristic were tested using

Table 4.1: Severity grading system for systemic hypersensitivity reactions

Grade	Criteria
I	Generalised urticaria (includes periorbital oedema), itching, malaise, anxiety
II	Angioedema, or two or more of: chest or throat tightness, nausea, vomiting, diarrhoea, abdominal pain, dizziness
III	Dyspnoea, wheezing or stridor, or two or more of: dysphagia, dysarthria, hoarseness, weakness, confusion, feeling of impending disaster
IV	Hypotension, collapse, loss of consciousness, incontinence of urine or faeces, cyanosis

Fisher exact, Spearman correlation and Mann-Whitney U tests for dichotomous, ordinal and continuous variables respectively. Statistical analyses were performed with SPPS ™ software version 11 and Analyse-it version 1.61 for Microsoft Excel ™.

4.3 Results

4.3.1 Population sting exposure and allergy prevalence

Four hundred seven of 560 households (73%) consented to participate. 1011 interviews (2.5 interviews per household) were performed, 52% were male and the median age was 37. This was comparable with 1996 Tasmanian census data. Sting exposure rates for each insect are presented in Table 4.2. There were no detectable gender differences in sting exposure. Not surprisingly, sting exposure rates were highest in the 15-65 year age group for each insect examined (all-insect sting exposure rates 23%, 27% and 16% in the <15, 15-65 and ≥65 age groups respectively).

Forty-six people reported systemic sting reactions. Thirty reported reactions to ant stings (27 to JJA, 3 to *M forficata*), 14 to honeybee and 6 to *V germanica*. Six people reported reactions to multiple insect species (mainly honeybee + JJA). Allergy prevalences for each insect, along with 95% confidence intervals and correlated with sting exposure rates are presented in Table 4.2.

For JJA allergic people, median age was 56 years (range 6-84) and 52% were male. Twenty-four clearly identified JJA as a cause and in another 3 where the insect was not clearly identified at the time of the sting, serum venom-specific IgE analysis implicated JJA with measured uptakes of 17-26%, but insignificant uptakes to other venoms. Ten

Table 4.2: Population sting exposure and allergy prevalence

Insect	Sting exposure (95% CI)		Stings per person exposed per year	Population allergy prevalence
	Lifetime *	Previous 12-mo		
Any insect	72% (69-75)	25% (22-28)	2.9	4.5% (3.5-6.0)
Any ant	53% (50-56)	16% (13-18)	2.3	3.0% (2.0-4.2)
JJA	40% (37-43)*	12% (10-14)	1.9	2.7% (1.8-3.9)
Honeybee	54% (51-57)*	7% (6-9)	1.3	1.4% (0.8-2.3)
V germanica	14% (12-16)	2% (1-3)	1.2	0.6% (0.2-1.3)
M forficata†	-	-	-	0.3% (0.1-0.9)

* Greater lifetime exposure to honeybee compared to jack jumper stings was repeated across all age groups. Thus, a larger proportion of the Tasmanian population is exposed to bee stings over a lifetime (possibly due to the greater range of flying insects), but jack jumper stings occur more frequently in areas where the ant is encountered. †Sting exposure data was not collected for *M forficata*.

experienced a grade III or IV reaction, a prevalence of 1.0%, (95%CI 0.5-1.8) for potentially life-threatening allergy.

JJA allergy prevalence was not affected by gender but increased with age (1.5% and 3.6% in the <35 and ≥35 age groups respectively, OR 2.44, 95%CI 1.02-5.8, p 0.05) and the presence of honeybee sting allergy (OR 16.9, 95%CI 4.9-58.0, p<0.001). Although honeybee sting exposure increased the risk of JJA sting exposure (OR 3.1, 95%CI 1.7-5.4, p <0.001), it was not significantly associated with an increased risk of JJA sting allergy (OR 1.06, 95%CI 0.2-4.6).

4.3.2 Emergency Department presentations

From the ED database, 264 Mueller grade I-IV sting reactions in 246 individuals were identified, attributed to JJA (43%, 95%CI 37%-49%), honeybee (23%, 95%CI 18%-29%), *V germanica* (5%, 95%CI 2.6%-8.3%), inchman (3%, 95%CI 1.6%-6.4%) and unidentified stings (25%). These proportions were unchanged when repeat sting reactions in the same individuals were excluded. One hundred fourteen JJA sting reactions occurring in 105 individuals, median age at presentation was 29 (range 1–78 years), and 60% were male (95%CI 50-69). 36% and 20% of cases were of grade III and IV severity respectively. Prior known JJA sting allergy was recorded in 54 (47%) of presentations. Patients with grade IV reactions tended to be older than those with less severe reactions (median ages 44 versus 24, p=0.047) but ranged in age from 1 to 78 years (5 were <15 years).

4.4 Discussion

4.4.1 Principal findings

JJA sting allergy prevalence in the Tasmanian community was 2.7% overall, double that for honeybee sting allergy. Annual sting exposure rates and ED presentations for each insect mirrored population allergy prevalences. The risk of JJA allergy was highest in those aged ≥35 and those with honeybee sting allergy. People experiencing hypotensive reactions were older than were those who did not.

4.4.2 Study strengths & weaknesses

Both parts of this study were exposed to the recollection and documentation biases inherent in retrospective studies. An attempt was made to minimise these effects by analysing relatively simple datasets. Although any study of accidental stings is hampered by lack of formal insect identification, the comparisons between different insects were facilitated by the main species being easily distinguished from one another (a honeybee, a wasp and a very distinctive ant). Specific IgE analysis was also used to identify causative insects where the clinical history was uncertain. Although asymptomatic sensitisation results in a poor positive predictive value (~0.1) if RAST is applied to the general population, in people with a history of sting anaphylaxis a high pre-test probability gives a high positive predictive value (≥0.9). This enabled accurate determination of the causative insect in a number of cases with unclear histories.

Another strength of this study was that people of all ages and residents of urban as well as rural areas were included in the random population survey, thus gaining a more accurate assessment of whole population allergy prevalence than studies which focus on higher risk adult &/or rural groups.

4.4.3 Comparison with related studies

Another group has also found that sting allergy prevalence is higher in older people.[168] JJA sting exposure in Tasmania is double that reported for adults in rural southeastern mainland Australia (Victoria) and the prevalence of allergy is higher (2.7% versus 1.9%).[28] The real difference between allergy prevalence is probably more than this, given that the Victorian study was restricted to adults living in rural areas– a higher risk group overall than this Tasmanian group that included urban populations and children who have a lower prevalence of sting allergy.

The overall sting allergy prevalence of 4.5% in this study, even though it included children and an urban population, is higher than the figures of 0.8-3.9% reported by other investigators.[187,188,190,193-196] Honeybee sting allergy prevalence in this study

(1.4%) was only slightly lower (probably not significantly so) than the 2.5% clinical allergy prevalence reported from the rural southwest of Western Australia.[190]

The finding that ED patients experiencing hypotensive reactions are significantly older, is consistent with mortality date for JJA and other insects finding deaths to be virtually confined to people over the age of 30 (Chapter 3: and 1.2.4.2 Table 1.4). A relationship between age and reaction severity has been noted previously, where people with hypotensive reactions to deliberate sting challenge were significantly older (mean 48 years for honeybee allergy) than those experiencing skin-only reactions (mean 28 years for honeybee allergy).[52]

Age effects may be due to the development of co-morbidities (although subjects in the study quoted above were excluded if any evidence of cardiac disease was found). Another explanation may lie with changes in cardiovascular homeostatic mechanisms with age. For example, people with a history of sting anaphylaxis have significantly reduced plasma renin, angiotensinogen, and angiotensin I & II as compared with healthy controls, with a significant inverse correlation between the severity of clinical symptoms and the plasma levels of these same mediators.[185] Although in that study patient age did not seem to have an influence on these mediator levels, age-related changes in the renin-aldosterone system in healthy people have been well documented previously, with plasma renin activity and plasma aldosterone levels being highest in the newborn, and lowest in the elderly population.[186]

4.4.4 Interpretation

The epidemiological data presented here indicates that the prevalence of JJA sting exposure and JJA sting allergy in Tasmania is excessive compared to mainland Australia. This effect appears to be additional to honeybee and *V germanica* allergies, leading to one of the highest reported population prevalences of sting allergy in the world. Tasmania's climate, cooler on average than mainland Australia, probably favours JJA. Tasmania's prominent agricultural economy also means that honeybee sting exposure is common.

Allergy prevalences are clearly linked to annual sting exposure rates and age, but not with lifetime exposure prevalence. Presumably, these observations are due to an increasing cumulative probability of encountering a TH2-type response with repeated exposure. The higher risk of JJA allergy in those with honeybee allergy (and visa versa) may be explained by the fact that people stung by honeybee are also more likely to be stung by JJA (and visa versa). However, the fact that JJA allergy correlated with the presence of honeybee allergy, but not honeybee stings, suggests (although is not

conclusive for) the existence of an individual predisposition towards sting allergy in general.

Older people appear to be at greater risk of severe reactions. However, potentially life-threatening reactions do occur occasionally in children.

4.4.5 Unanswered questions

Although this retrospective analysis indicates a clear relationship between age and JJA reaction severity, a prospective analysis of accidental stings is required to confirm the clinical utility of this and other factors to identify those most at risk.

Chapter 5: Initial assessment and prospective follow-up of JJA sting allergic volunteers

5.1 Introduction

As a prelude to testing the efficacy of VIT for JJA allergy, this section aimed to determine the clinical features and natural history of JJA sting allergy, and to determine any predictors of severe systemic reactions in a prospective study of a large cohort of allergic people. A secondary aim was to assess the diagnostic utility of serum sIgE (RAST) to identify people with a history of definite JJA sting allergy. The Royal Hobart Hospital Ethics Committee granted ethical approval for the study.

5.2 Methods

5.2.1 Allergic volunteers; clinical features and serum IgE analysis

JJA sting allergic volunteers were recruited from the Royal Hobart Hospital ED study, population survey, local doctors, and media coverage from September 1998 onwards. Structured interviews recorded demographics, "cardiovascular co-morbidity" (hypertension, ischaemic heart disease or heart failure), "airways disease" (asthma or chronic airflow limitation) and for each insect type causing a reaction; age at first systemic reaction, number of reactions requiring medical care, number of reactions treated with adrenaline, severity of worst reaction according to the system outlined in Table 4.1, and a detailed description of individual reaction features for the most recent sting. Interview results were checked against available medical, nursing and ambulance records and entered directly into a customized relational database designed to store all epidemiological and patient contact data (Microsoft Access).

If subjects consented and were able to provide serum, samples were sent to the Kolling Institute Molecular Immunology Unit, Royal North Shore Hospital Sydney (Dr Brian Baldo, Qi Xuan Wu), for sIgE analysis by RAST.[27,381] Results were expressed as percentage uptake of anti-IgE to serum IgE bound to venom on a solid phase. JJA venom was used in all cases and for the first 183 subjects the following additional venoms were used; honeybee, yellow jacket (*Vespula* spp.), and a panel of gulosa species group venoms closely related to *M forficata* (*M gulosa*, *M pyriformis*, *M tarsata* and *M simillima*). *M forficata* venom was not available at the time of performing these analyses. The limitation on the number of sera tested against an extended panel of venoms, as well as an inability to obtain detailed data on binding to synthetic peptide

allergens, was imposed by a disruption and eventual cessation of research activity at the Kolling Institute Molecular Immunology Unit.

To evaluate diagnostic performance, 149 control sera taken from non-allergic patients attending the Royal Hobart Hospital ED and requiring blood to be taken for unrelated reasons were tested for IgE reactivity against JJA venom. Questionnaires were completed for each control subject with regard to previous lifetime and 12 month sting exposure. Patients with severe multi-system disease, major injuries, life-threatening illness, immunological disease and haematological malignancies were excluded from the control group.

5.2.2 Accidental field stings

Structured datasheets and reply-paid envelopes were provided to monitor subsequent stings. The datasheets contained prompts for all significant symptoms as outlined in our grading system (Table 4.1). Reaction details were confirmed with medical, nursing and ambulance records if treatment was provided. Regular telephone calls were made to detect unreported stings. Termination of follow up was defined by the last successful telephone contact (last attempts made in May 2002) or at entry into our venom immunotherapy trial between August 2001 and November 2001.

5.2.3 Statistical analyses

For the analysis of reaction severity, dichotomous outcome variables were defined by reaction occurrence, occurrence of a grade III-IV (severe) reaction, or occurrence of a grade IV (hypotensive) reaction. Associations between each of these outcomes and baseline characteristics were tested using Fisher exact, Spearman correlation and Mann-Whitney U tests for dichotomous, ordinal and continuous baseline variables respectively. Associations between prior reaction grade (I-IV) and subsequent reaction grade (I-IV) were tested using Spearman correlation. Statistical analyses were performed with SPPS ™ software version 11 and Analyse-it version 1.61 for Microsoft Excel ™.

5.3 Results

5.3.1 Baseline clinical characteristics

Three hundred eighty-eight volunteers were recruited with a median age of 51 years (range 2-90). Fifty percent were male and 102 (26%) reported a cardiovascular co-morbidity; 85 people had hypertension, 32 ischaemic heart disease, and 9 heart failure.

Sixty-one (16%) had airways disease. Three hundred eighty-one reported a systemic reaction following a clearly identified JJA sting and in a further 7 who could not identify the causative insect, serum venom-specific IgE analysis implicated JJA as the cause with tracer uptakes of 3-26% but insignificant uptakes to other insect species. Fifty percent reported systemic reactions on more than one occasion. Thirty (7.7%) gave a history of multiple sting allergies (honeybee 20, *V germanica* 7, inchman 5).

5.3.2 Prior reaction severity; correlation with other baseline clinical characteristics

In those experiencing a systemic reaction when last stung the proportion of grade IV reactions was 9% (10/116) for those aged <35 at the time of the sting, versus 22% (47/217) for age ≥35, OR 2.9 (95%CI 1.4-6.0, p 0.002). The chosen cut off age (35) for this analysis was determined by examining the centred moving average for the proportion of grade IV reactions by age (Figure 5.1). This analysis suggested a marked increase in the proportion of grade IV reactions at around this age, although a linear relationship between age and the risk of a grade IV reaction could not be excluded. Risk of a grade IV reaction at any stage in the past was not significantly influenced by gender, comorbidities, or number of prior reactions. Similarly, the proportion of grade III reactions (or grade III/IV reactions combined) was not influenced by age, gender or comorbidities, however the presence of respiratory disease increased the risk of bronchospasm (OR 2.1, 95% CI 1.02-4.45, p 0.05).

5.3.3 Serum JJA venom-specific IgE

Sera from 275 allergic patients with a definite history of anaphylaxis to JJA were analysed for IgE reactivity with JJA venom by RAST. Ninety-three percent were reactive (>0.3% tracer uptake) to JJA venom, versus 22% of 149 non-allergic control sera tested (sensitivity 0.93, specificity 0.78). Using the more traditional RAST tracer uptake cut offs of 1% and 2%, sensitivities/specificities were 0.79/0.85 and 0.62/0.92 respectively. A receiver-operator characteristics (ROC) curve is presented in Figure 5.2, which demonstrates that improved sensitivity with minimal loss of specificity can be achieved if a lower diagnostic threshold is used. Conversely, high tracer uptakes of >7.5% appeared to have a high specificity (0.99) but low sensitivity (0.34) for identifying people with a history of clinical allergy.

Fifty-four people in the non-allergic control group could not recall any exposure to JJA or any other ant; IgE reactivity was <0.3% in all except two. In the 79 non-allergic people who could recall a history of exposure to JJA, 12/17 (71%) who could recall a sting in the previous 12 months had detectable IgE reactivity to JJA venom, versus

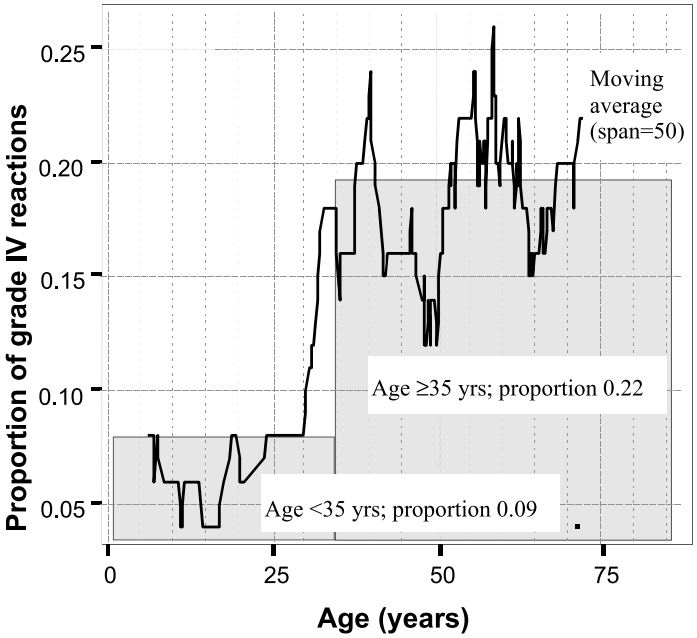
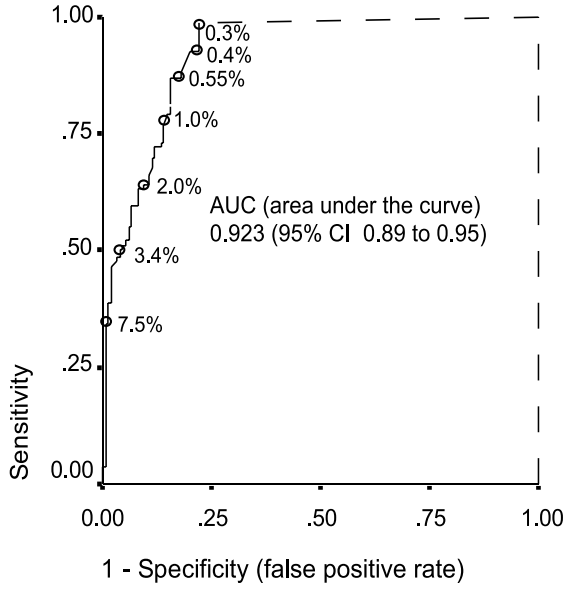


Figure 5.1: Centred moving average for the proportion of grade IV reactions by age



Explanatory note:

ROC curves plot the performance of each possible diagnostic cut-off (the values that are plotted along the curve) in terms of *sensitivity* (number of true positives divided by the number with the disease) and *specificity* (number of true negatives divided by the number without the disease).

The greater the area under the curve, the better the performance of the test.

Figure 5.2: Receiver-operator characteristics (ROC) curve for JJA sIgE RAST (expressed as % tracer uptake) using history of a systemic reaction (grade I-IV) as the diagnostic gold standard

16/62 (26%) who had been last stung more than 12 months previously (p = 0.001). This association between detectable reactivity and time from last sting was not evident in the allergic group, where 73/77 (95%) and 185/199 (93%) of those exposed within 12 months and more than 12 months ago respectively, had detectable IgE reactivity to venom. However, the median tracer uptake values were higher in those stung within 1 year (5.7%) compared to those stung more than 12 months previously (2.8%), p = 0.003.

5.3.4 Serum venom-specific IgE to other Myrmecia, bee and wasp venoms

Testing against all available venoms was done for the first 183 JJA allergic volunteers; ≥1.0% tracer uptake against gulosa group, honeybee and wasp venoms was apparent in 46 (25%), 30 (16.4%) and 21 (11.5%) respectively. Only one person with no detectable reactivity to JJA had reactivity to another venom (gulosa group, specifically *M pyriformis,* which is closely related to, and often confused with, *M forficata*); this person had experienced multiple severe (grade III-IV) systemic allergic reactions to both JJA and *M forficata*.

A plot of % tracer uptake for JJA versus gulosa group venoms is presented in Figure 5.3, showing two distinct patterns:

- Sera with moderate-high JJA reactivity also showing low level reactivity to gulosa group venoms (probably antibody cross-reactivity), and;

- A smaller number of sera that recognise gulosa group venoms to a greater extent than JJA.

Of 27 JJA allergic people with >2% tracer uptake against gulosa group venoms, 20 (74%) had >2% tracer uptake to more than 1 gulosa group venom. Of these 20, 17 displayed some degree of tracer uptake to every gulosa group venom. Two had experienced a reaction either to other ants (probably *M forficata*). Another had experienced reactions to unidentified insects.

Twenty-seven people who were clinically and serologically reactive to JJA had no recollection of honeybee or *V germanica* sting exposure. In this group, IgE reactivity to honeybee venom was identified in 1 and *Vespula* spp. venoms in 2.

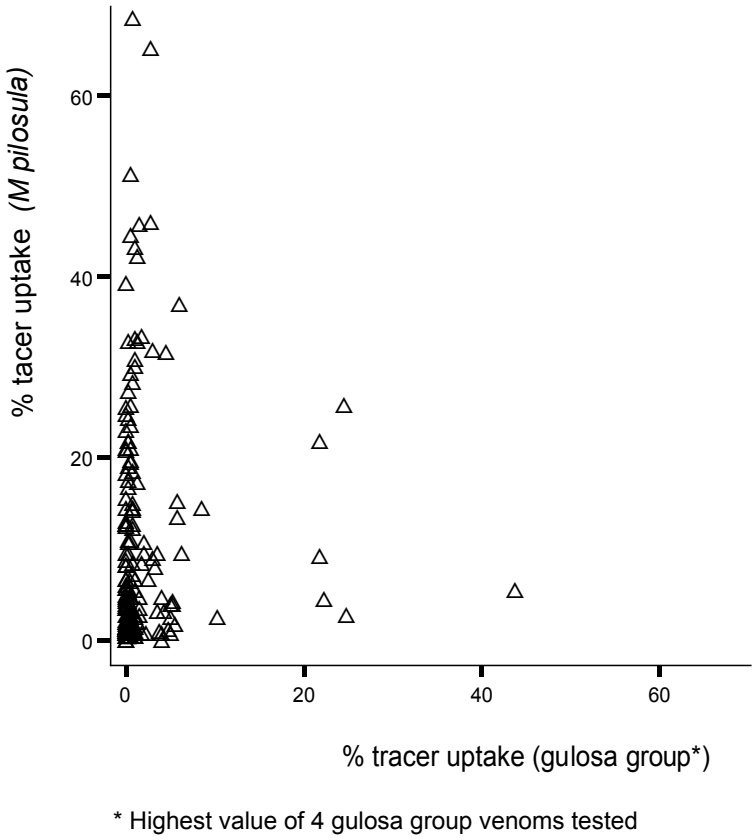


Figure 5.3: Dot plot of individual serum JJA sIgE versus gulosa group sIgE

5.3.5 Accidental field stings

5.3.5.1 Field stings during the follow up period

Twenty people were lost to follow up, leaving 368 for analysis. Mean follow up interval was 2.3 years. Eighty-seven volunteers experienced 113 clearly identified JJA stings causing 79 systemic reactions in 60 people, a reaction rate of 70% (95%CI 61-78). Median age of those stung was 59 years (range 3-86), 42% were male (95%CI 32-51) and 31% reported cardiovascular co-morbidity; 23 people had hypertension, 8 ischaemic heart disease, and 2 heart failure. Twelve had airways disease.

Systemic reactions were more common in women (OR 3.7, 95% CI 1.4-9.7, p 0.01), people treated with adrenaline for their most recent sting (OR 5.7, 95%CI 1.5-21.1, p.006) and those with a history of severe reactions as defined by our grading system (grades III and IV). Prior reaction severity was the only statistically significant

Table 5.1: Worst reaction during follow-up period versus severity of worst prior reaction

Worst prior reaction at time of first interview	No. exposed after first interview	Systemic Reaction (%)*	Grade of worst reaction during follow-up †			
			I	II	III	IV
Grade I	12	7 (58%)	5	1	1	
Grade II	16	9 (56%)	6	3		
Grade III	40	28 (70%)	10	5	12	1
Grade IV	19	16 (84%)	2	2	7	5

* p = 0.04 (combining grades I and II to form a three-tiered severity scale)

† Spearman correlation coefficient = 0.42 (p < 0.0001)

Table 5.2: Worst reaction during follow-up period versus severity of most recent sting reaction

Most recent sting at time of first interview	No. exposed after first interview	Systemic Reaction (%)*	Grade of worst reaction during follow-up †			
			I	II	III	IV
No reaction	13	6 (66%)	3		1	2
Grade I	21	16 (76%)	8	3	4	1
Grade II	15	9 (60%)	5	4		
Grade III	26	19 (73%)	7	3	9	
Grade IV	12	10 (83%)		1	6	3

* p 0.06 (combining grades I and II to form a three-tiered severity scale)

† Spearman correlation coefficient 0.28 (p<0.01)

predictor of subsequent reaction severity; worst reaction severity (Table 5.1) was a better predictor than recent reaction severity (Table 5.2). Only 3 people experienced a reaction during follow-up that was more severe than the worst prior reaction at the time of study entry.

The six grade IV reactions occurred in people aged ≥35, whereas none of the 16 people in the <35 age group had a grade IV reaction. However, this difference did not reach

significance (p=0.587). Although gender correlated with reaction occurrence, it was not predictive of severity. Serum venom-specific IgE levels (Figure 5.4), duration of known allergy, number of reactions, time elapsed since last sting and co-morbidities did not predict either the occurrence or severity of reactions during the follow up period.

5.3.5.2 Pooled descriptive analysis of sting episodes

Details from 414 reactions –those occurring with the most recent sting episodes at study entry and during the follow up period– are presented in Table 5.3. Reactions occurred to single stings in 76%. The maximum number of simultaneous stings was 35. Adrenaline, steroids and antihistamines were administered in 41%, 20% and 63% respectively. Reaction severity correlated significantly with adrenaline usage, 14.6%, 39.8%, 48.7% and 69.2% for grades I-IV respectively (Spearman correlation coefficient 0.36, p<0.0001). There were 5 delayed phase reactions (1.2%); 3 urticarial, 1 dyspnoea/cough and 1 episode of generalised oedema, aches and pains for several days after 10 simultaneous stings.

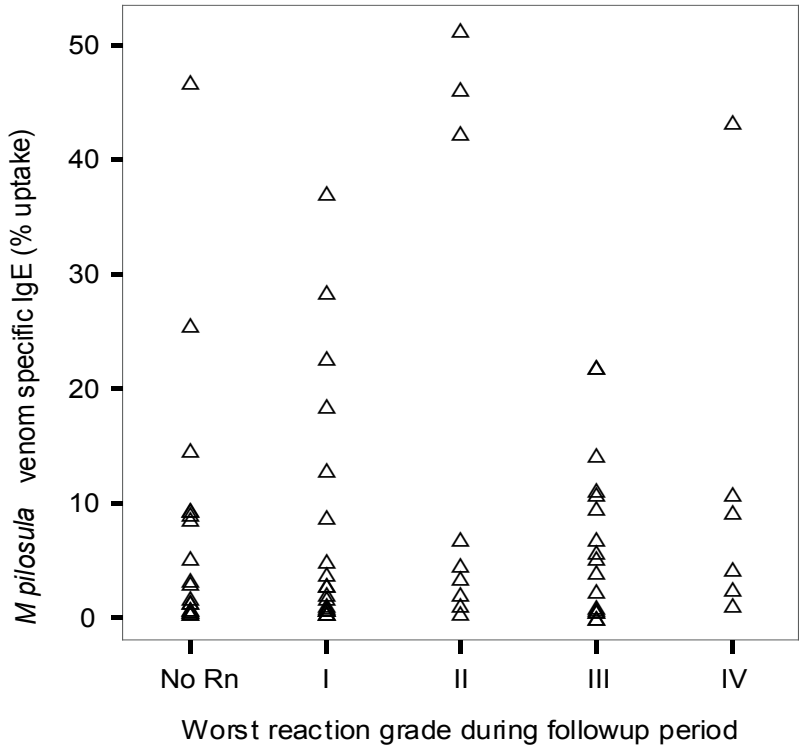


Figure 5.4: Serum JJA sIgE (% tracer uptake) at study entry versus severity of worst sting reaction during prospective follow up period

Table 5.3: Reaction features for 414 *M. pilosula* sting anaphylaxis episodes in allergic volunteers, grouped according to reaction grade

Grade I features	Urticaria 45.2%, periorbital oedema 22.5%, itch 56.8%, malaise 18.8%, anxiety 24.4% 80% reported one or more skin features (urticaria, itch, periorbital oedema or angioedema)
Grade II features	Angioedema 41.5%, chest tightness 26.6%, throat tightness 30.4%, nausea 16.4%, vomiting 11.4%, diarrhoea 5.1%, abdominal pain 6.8%, dizziness 20.5%
Grade III features	Dyspnoea 39.1%, wheezing 12.1%, stridor 1.7%, dysphagia 12.8%, speech difficulties 15.5%, weakness 15.0%, confusion 4.6%, feeling of impending disaster 12.6%
Grade IV features	Documented hypotension 8.7%, collapse 9.2%, loss of consciousness 6.0 %, incontinence of urine or faeces 1.7%, cyanosis 0.7%

Breakdown by reaction grade: Grade I, 103 (24.9%); Grade II, 88 (21.3%); Grade III, 158 (38.2%); Grade IV, 65 (15.7%)

5.4 Discussion

5.4.1 Principal findings

The individual features of systemic reactions to JJA stings were typical for anaphylaxis, and single stings precipitated the majority (76%) of systemic reactions. Delayed ("biphasic") reactions were rare. RAST had useful sensitivity (0.9) and specificity (0.8) for the diagnosis of JJA allergy using a low tracer uptake threshold (>0.3%), but non-allergic people with recent (<12 months) sting exposure had a high rate of positive RAST (71%). There appeared to be significant IgE cross-reactivity with other *Myrmecia* venoms, but not with honeybee or wasp venoms.

Using the modified Mueller severity scale, reaction grades correlated well with adrenaline usage. Those ≥35 at the time when last stung had a greater risk of hypotensive reactions (OR 2.9). Asthma also appears to modestly increase the risk of bronchospasm if an allergic reaction occurs. During follow-up, 79 (70%) of 113 jack jumper stings caused systemic reactions. Only prior worst reaction severity predicted the severity of follow-up reactions, with the majority experiencing similar or less severe reactions when stung again.

5.4.2 Study strengths & weaknesses

As for the previous chapter, any research examining clinical sensitivity to accidental stings is hampered by lack of formal insect identification. Nevertheless, the JJA is a distinctive insect and analysis (RAST) for the majority of people in the study was able to confirm our clinical assessment of their sting allergy.

Strengths of this study were that: (i) The accuracy of retrospective data was maximized by cross-referencing patient recall with medical notes wherever possible; (ii) Reaction grade corresponded closely to treatment with adrenaline, indicating that the grading system was a useful marker of severity, and; (iii) The prospective design for the analysis of accidental field stings, including prompt follow up with treating health professionals, minimised the effect of recollection and selection biases when analysing changes in sting reaction severity over time.

The low prevalence of severe comorbidities such as heart failure and ischaemic heart disease in allergic volunteers, as well as possible under-recognition of these conditions, reduced the power of this study to detect an effect of these factors on reaction severity. The very small number of grade IV reactions in the prospective cohort of accidental stings, combined with under-representation of people aged <35, meant that the prospective part of the study lacked adequate power to conclude that any certain clinical characteristic does not contribute to reaction severity. However, from a clinical perspective, any feature not sufficiently predictive of reaction severity to be detected in a study of over 100 accidental stings is unlikely to be useful when deciding to whom adrenaline auto injectors and VIT should be offered.

5.4.3 Comparison with related studies

5.4.3.1 Serum venom-specific IgE analysis

These results are consistent with a number of studies of honeybee and wasp venom allergy that have found a significant proportion (as high as 25%) of a population may show evidence of sIgE without any evidence of clinical sensitivity either retrospectively or in response to subsequent stings.[188,225-227,230] This leads to poor specificity if attempts are made to use serum IgE analysis to diagnose allergy in the general population.

A decline in IgE reactivity to venoms over time since being stung has been noted previously in non-allergic people exposed to wasp stings, with the authors suggesting venom-allergic people may differ by being more prone to exhibit sustained sIgE responses.[192] This fits with the finding here of a time-effect on sIgE in the non-allergic group that was less apparent in the allergic group.

IgE cross-reactivity between bees and wasp venoms is a well-recognised phenomenon.[396] Significant cross reactivity of IgE antibodies to fire ant venom (specifically the allergen *sol i 1*) with honeybee and wasp venoms (and visa versa) has also been identified,[397] whereas the result here do not show any evidence of cross-reactivity between JJA and honeybee or wasp venoms. Cross-reactivity of JJA allergic

sera with other *Myrmecia* venoms has been noted previously, although that study was limited by a lack of correlation of serological findings with clinical reactivity.[27]

5.4.3.2 Reaction features and predicting reaction severity

The individual reaction features reported here are similar to those reported from allergic reactions to other Hymenoptera.[34-38] Notably however, JJA stings appear to be potent allergenic stimuli; the 70% follow-up reaction rate found here exceeds the 27-57% reaction rates reported by large prospective studies of honeybee and wasp allergies.[52, 198,245,278,398-400] The incidence of protracted or biphasic reactions to Hymenoptera stings has not been specifically addressed previously, although reports of severe protracted/ biphasic reactions appear to be confined mainly to food allergy.[53,54]

Fluctuation in reaction severity between stings has been attributed to the variable amount of venom delivered.[109] Despite the well recognised fluctuation in severity from sting to sting, one study of 324 honeybee and yellow jacket provocation tests found no reaction to be more severe than the worst previous reaction, suggesting that each individual person has a tendency towards reacting in a certain way, or with a certain maximum reaction severity.[52] However, another large study of honeybee and yellow jacket allergy, although finding a similar pattern, did identify a number of people (10% of those allergic to honeybee stings) who proceeded to have more severe reactions than previously when they were deliberately stung.[198]

Although the initial assessment of allergic volunteers, along with the analysis of ED presentations (Chapter 4:) confirms a relationship between age and reaction severity that is consistent with prior studies,[52] this effect was apparent but not statistically significant in the prospective follow up arm because the small number of younger volunteers lead to an inadequate contrast between older and younger people.

5.4.4 Interpretation

Prior worst reaction severity and age over 35 years are the most important predictors of severe (hypotensive) sting reactions, and may be used to guide the application of VIT. However, a degree of caution is required because although most people will have reactions that are of similar severity (or milder) when stung again, some people will have more severe reactions, as was the case in this study. This is to be expected given that initial reactions may follow smaller stings (less venom injected) with that persons' "maximum reactivity" not being evident until a later, more potent sting. It is also possible that in some people, reactivity increases with successive stings.

Although not finding evidence to support an effect of co morbidities or regular medication on the risk of a severe reaction, the analysis was underpowered in this regard, and although not major determinant of reaction severity, these factors may still contribute to death when a grade IV reaction occurs.

Using an appropriate diagnostic threshold, detection of sIgE by RAST appears to be sensitive for JJA allergy, but lacks specificity. IgE cross-reactivity may lead to anaphylaxis after the sting of ant species not previously encountered. Because of the geographical distributions of the species against which sera from this study were tested (none of which are found in Tasmania), it is certain that the majority of participants in the study had never encountered all the venoms with which their IgE reacted. This indicates significant antibody cross-reactivity between *Myrmecia* venoms rather than separate sensitisations. Although no studies of IgE cross-reactivity between *Myrmecia* and other Hymenoptera genera have been published, the unique peptide structure of JJA venom suggests that such cross-reactivity would be unlikely. The data presented here supports this theoretical prediction, but lacks statistical power because of the small number of people who were JJA allergic but could not recall previous exposure to honeybee and *V germanica* stings.

5.4.5 Unanswered questions

Because the follow-up cohort was underpowered to identify an effect of age on reaction severity, it was not possible to determine whether age and prior reaction severity are independent predictors of reaction severity. A much longer period of follow up would be required to determine whether advancing age might lead to reactions that have previously been relatively mild, becoming more severe. The contribution of co morbidities towards reaction severity and mortality also remain to be defined.

Although a useful diagnostic test to determine likely causation of prior sting reactions, sIgE appears to be of little value predicting subsequent reaction severity to accidental stings in the setting of JJA allergy. The nature of IgE cross-reactivity between *Myrmecia* species is also obscure, specifically with regard to common peptide sequences/structures and the potential for cross-reactive antibodies to trigger reactions to ants encountered for the first time. These issues will be of greater clinical importance in parts of Australia where multiple *Myrmecia* species are encountered.

PART III: VENOM ANAPHYLAXIS AND IMMUNOTHERAPY

Chapter 6: Introduction

6.1 Clinical trial of venom immunotherapy

As outlined in Chapter 2: (Research objectives and outline), Part III of this thesis reports a randomised, double blind placebo-controlled, crossover clinical trial that aimed to test the tolerability and efficacy of outpatient VIT for preventing JJA sting anaphylaxis. The primary outcome measure was response to a deliberate sting challenge.

6.2 Ancillary studies

The inclusion of a placebo arm, with inevitable episodes of anaphylaxis to deliberate sting challenges, presented a unique opportunity to examine the clinical and biochemical features of JJA sting anaphylaxis induced in a controlled environment, including assessment of a resuscitation protocol. The inclusion of a placebo arm also enabled assessment of a set of *in vitro* laboratory tests for their diagnostic utility to predict clinical sensitivity in untreated individuals (with the potential to select those most likely to benefit), and provided an ideal control group to assess these same laboratory tests for their potential as markers of successful VIT.

6.2.1 Sting anaphylaxis; clinical observations, management and diagnosis

As described in the preceding literature review (1.2.2) human studies of pathophysiology are relatively sparse and by necessity, opportunistic. Furthermore, although adrenaline is an accepted (and anecdotally effective) treatment for anaphylaxis, little supportive human data is available. There have been recurrent debates over the appropriate route of administration and indications for treatment with adrenaline. Commentaries emphasise the need to adapt to prevailing clinical conditions; clinical urgency, degree of circulatory compromise, availability of vascular access, and the level of care available.[96,143,401]

Once emergency treatment has been given, diagnostic issues need to be addressed. Anaphylaxis is an important differential that can be difficult to confirm in some clinical situations. Examples where anaphylaxis may be under-recognised include unwitnessed sudden death, cardiovascular collapse in the absence of other signs of anaphylaxis, and acute asthma. At the other extreme, scombroid fish poisoning may present in a manner similar to anaphylaxis and be misdiagnosed as seafood allergy.

Anecdotally, less experienced clinicians may also misinterpret clinical presentations, erroneously diagnosing anaphylaxis as a vasovagal collapse, or an atypical dystonic drug reaction (with tongue and vocal cord dystonia misinterpreted as submandibular swelling and laryngeal oedema) as anaphylaxis. From a perspective of drug reaction surveillance, the aetiology of radiographic contrast (and other drug) reactions can at times be obscure and require investigation to look for the possibility of anaphylaxis.[402] Serum mast cell tryptase is a widely available test that the UK Resuscitation Council recommends to confirm the diagnosis of anaphylaxis.[140]

The aims of this ancillary study were therefore to describe the clinical features of sting anaphylaxis, assess a treatment protocol consisting of supplemental oxygen, a carefully titrated intravenous adrenaline infusion, and volume resuscitation, and to determine the diagnostic performance of tryptase measurements in identifying anaphylaxis.

6.2.2 Predicting sting challenge reaction risk

Current practice is to offer VIT to people with a history of severe systemic reactions and positive intradermal venom skin tests. However, the rational application of VIT is hindered by the fact that only 25-50% of people with a history of systemic sting reactions and positive skin tests react to deliberate sting challenge.[52,198] Therefore, the majority of people undergoing VIT do not require this treatment.

Only prior reaction severity and older age appear to distinguish those people at particular risk.[52] Any test used to exclude people from receiving VIT, or from carrying adrenaline following VIT, would need to be close to 100% sensitive for identifying those at risk of a severe reaction. Various basophil activation and mediator release tests are now commercially available,[403] and there has been strong support for use of the basophil activation test (BAT) in allergy diagnosis.[404] However, assessments of sensitivity and specificity for venom allergy diagnosis have been limited to comparisons with clinical history and skin tests. Given that intradermal tests correlate poorly with clinical sensitivity,[52] such studies provide little rationale for changing current practice.

This ancillary study aimed to assess the potential utility of available *in vitro* tests (namely venom-induced leukocyte proliferation, cytokine excretion, basophil activation, histamine and leukotriene release tests), for excluding low risk patients from receiving VIT.

6.2.3 Changes in laboratory parameters with VIT

VIT failure rates ranging from 0% to 40% have been reported (see literature review, 1.5.1.2). Currently, the only way of detecting VIT failure, other than noting problems in tolerating injections, is to perform a sting challenge. From a clinical logistical perspective, an *in vitro* assay would have many advantages. From a research perspective, it would also be useful to be able to measure laboratory parameter changes when developing novel immunotherapies, rather than exposing participants to a deliberate sting challenge.

This ancillary study was therefore designed to identify *in vitro* tests that showed significant changes during VIT (in comparison to a matched placebo group) and that might therefore have potential as indicators of successful VIT.

6.3 Ethical issues

6.3.1 Use of a placebo group

The inclusion of a placebo group was considered essential because without demonstrating an ability to precipitate severe anaphylaxis, it could not be certain that the sting challenge was an adequate test of immunotherapy. Failure to rigorously determine efficacy of immunotherapy could lead to a false sense of security in patients. This was a contributing factor in one of the deaths reported in Chapter3, of a man who erroneously believed that he was protected by WBE immunotherapy. Furthermore, the natural history of sting allergy is unpredictable, and many people (as many as 75% in the case of wasp sting allergy) lose their sensitivity, as measured by a deliberate sting challenge.[52,198] Therefore, a concurrent control group was required for statistical analysis, and because no other treatment was available for comparison, this control group had to be placebo treatment.

Although workers in one small series reported several serious reactions to sting challenge,[39] no health or age-based exclusion criteria seem to have been applied, and participants had been exposed to large test doses of venom antigen immediately before the sting challenge, which probably contributed to the severity of reactions. In two large sting challenge studies using strict exclusion criteria to minimise risk, 803 people have been deliberately stung prior to receiving VIT without any reported adverse event,[52,198] even if severe reactions were allowed to resolve without the use of adrenaline.[40] This indicated that a placebo-controlled sting challenge trial would be safe if the same exclusion criteria were applied, with the potential long-term benefit

to participants of developing a proven immunotherapy likely to outweigh the small additional risk imposed by the trial.

6.3.2 Determining reaction severity

When determining the result of a diagnostic sting challenge, an accurate measure of clinical reaction severity is required. If for example, a mild reaction in the VIT is treated with adrenaline before severe manifestations arise, then the investigator might erroneously conclude that the treatment was efficacious in that individual. Conversely, the same early treatment in the placebo group might lead to an inability to be certain that the sting challenge procedure was an adequate test of efficacy.

Therefore, it was important to leave sting reactions untreated until the patient reached objectively defined stages of respiratory or cardiovascular compromise. Because cardiorespiratory compromise is the generally accepted threshold for treatment with adrenaline,[139-141] such an approach was considered to be consistent with current treatment of allergic reactions and not to carry substantial additional risk, especially because sting challenges were performed in a critical-care environment.

6.3.3 Double blinding

Double blinding of immunotherapy was necessary because many sting reaction features could be open to subjective interpretation and because patient anxiety might have affected the results.

6.4 Ethics approval

Both the Royal Hobart Hospital and Flinders Medical Centre ethics committees approved the project. Although it was initially planned to perform the trial at both sites, regulatory issues resulted in the trial being confined to within the Tasmanian public hospital sector. The Royal Hobart Hospital ethics committee approved two trial sites, namely the Departments of Emergency Medicine at the Royal Hobart Hospital and the Northwest Regional Hospital Burnie.

Chapter 7: Methods

7.1 Trial participants

Volunteers were recruited from a mail out to participants in our Epidemiological study (Chapter 5:) and from local media coverage. Patients were considered for the trial if all of the following conditions were satisfied at the time of initial telephone contact; grade II–IV hypersensitivity (Table 4.1) to a clearly identified or presumed JJA, age between 17 and 65 years, no history of hypertension, heart disease, or poorly controlled lung disease, not receiving ACE-inhibitor or β-blocker therapy, and willing to undergo a sting challenge with the risk of potentially life-threatening anaphylaxis.

Volunteers were then assessed by a structured interview, physical examination, electrocardiography and spirometry to confirm their eligibility according to the above criteria. After fully informed written consent was obtained, clearly outlining the risks of immunotherapy and sting challenge, eligible people then underwent intradermal skin testing (see 7.3 below). Inclusion in the clinical trial required a positive JJA venom skin test. If results of the skin test were negative, the skin test was repeated before declaring a negative result. People with negative skin tests, although excluded from this study, were advised of the potential risk from future stings and were provided with appropriate action plans including injectable adrenaline where appropriate.

Mast cell tryptase concentration was determined at trial entry for each participant using a fluoro enzyme immunoassay (Unicap Tryptase, Pharmacia and Upjohn, normal range <12 μg/L) to screen for the presence of mastocytosis, reported to be more common in people with severe sting allergy.[202] Baseline sIGE (RAST) against JJA venom were performed for all participants as described in 7.6.2 below.

7.2 Venom extracts

JJA venom used for skin testing, immunotherapy and all *in vitro* analyses was obtained from a single large batch prepared locally by venom sac dissection from JJA gathered at various locations around Tasmania. After rupturing sacs with a tissue homogeniser, the venom was filtered through a 0.22-micron low protein-binding filter, lyophilised and weighed, dispensed into aliquots, then stored at -20 ºC until required.

For human diagnostic and therapeutic use, venom was dispensed into vials, each

containing 50 or 120 μg of venom protein and 20 mg mannitol. These were lyophilised again, then vacuum-sealed and stored below 8°C. An identical approach was used to prepare venoms for the other two local *Myrmecia* species (*M forficata* and *M esuriens*). Honeybee and European wasp venoms, standardised and registered for diagnostic and therapeutic use, were purchased from Bayer Australia.

7.3 Venom skin testing

Venom skin tests (VST) were performed by intradermal injection of about 0.02 mL of venom solution to form a 3-5 mm bleb, concurrently with positive control (histamine) and negative control (normal saline) solutions. A positive result was defined by wheal growth of at least 2 mm plus a flare greater than or equal to 10 mm at 20 minutes. An initial concentration of 0.01 μg/mL was used, increasing to 0.1 μg/mL and then 1.0 μg/mL to determine the venom concentration threshold giving a positive result. Testing was performed using JJA venom, and a panel of other insect venoms encountered locally (*M forficata*, *M esuriens*, honeybee and European wasp). Information from skin testing against the full range of local venoms was done in order to aid clinical interpretation of likely causation when the identity of an insect causing a reaction was unclear.

7.4 Immunotherapy

The trial was conducted in the EDs of two Tasmanian public hospitals, the Royal Hobart Hospital and North West Regional Hospital, Burnie. In case of accidental stings during the trial, all participants in the trial were provided with written action plans, Epipen and/or ampoules of adrenaline along with instruction in their use.

Initial trial arms (venom versus placebo) were commenced in August-November 2001 (spring and early summer). The crossover treatment phase for participants initially given placebo was commenced in Jan 2002 (mid summer) and most sting challenges post crossover were completed by the end of autumn and the last sting challenges were completed before the end of winter (August 2002).

7.4.1 Placebo-controlled randomised phase

All participants were initially allocated to treatment with either venom or placebo. An investigator not involved in direct patient care (Michael Wiese) used a random number-based sequential allocation process to allocate participants to treatment

Table 7.1: Outpatient semi-rush hyposensitisation regime

	Amount of venom (μg)		
Visit 1	0.0001	0.001	0.01
Visit 2	0.01	0.1	0.3
Visit 3	0.3	1	3
Visit 4	3	10	
Visit 5	10	20	
Visit 6	20	30	
Visit 7	30	40	
Visit 8	50	50	
Visit 9	70	30	
Maintenance	100		

Values are given in micrograms. Injections were given at 20-60 minute intervals. The first dose was at 1/10 the concentration at which intradermal skin testing was positive. This regime was used as a guide only, adjusted according to patient tolerance. Visits 1-10 were 5-10 days apart, then gradually extended to monthly maintenance visits. The minimum period of observation following the last injection of each visit was 1 hour.

groups. Patient-specific treatment packs were dispensed, containing either lyophilised venom or placebo and a diluent solution of 0.05% polysorbate 80. Packs and contents were identical in appearance. In placebo vials, 100 μg venom protein was substituted with 7 μg or 8·4 μg histamine acid phosphate to simulate the anticipated variability in local reactions.

VIT was given in accordance with an outpatient "clustered" or "semi rush" procedure (Table 7.1), choosing an arbitrary maintenance dose of 100 μg, based on the maintenance dose used in honeybee and wasp VIT. The first dose was determined by the threshold at which patients were positive to intradermal skin testing- i.e., 0.1 mL of a solution at a tenth of the concentration that produced a positive skin test. For example, 0.00001 μg for participants who had a positive skin test at 0·001 μg/mL. This regimen was used as a guide only, and was adjusted in accordance with patient tolerance. Participants were observed for at least 1 h after the last dose was given. Patients who had adverse reactions were allowed to take prophylactic oral antihistamines (10 mg loratadine with 300 mg ranitidine taken 2 h before immunotherapy).

Table 7.2: Clinical trial systemic reaction severity grading system

Grade	Criteria
I	Generalised urticaria (includes periorbital oedema), itching, malaise, anxiety
II	Angioedema, or two or more of: chest or throat tightness, nausea, vomiting, diarrhoea, abdominal pain, dizziness
III	Dyspnoea, wheezing or stridor, or two or more of: dysphagia, dysarthria, hoarseness, weakness, confusion, feeling of impending disaster
IV	Hypotension*, collapse, loss of consciousness, incontinence of urine or faeces, cyanosis

* For the purposes of reactions to immunotherapy and sting challenge reaction grading, hypotension with a systolic blood pressure <90 mmHg was required to define a grade IV reaction. For retrospective analysis of reaction severity, documented hypotension was not required.

Immediate-type hypersensitivity reactions to immunotherapy were graded according to the system outlined in Table 4.1 but with the following modifications: at least one objective physical sign was needed to define a systemic reaction of any grade; and grade IV reactions were assigned only when systolic blood pressure was less than 90 mm Hg (Table 7.2).

7.4.2 Crossover phase

To avoid continued ineffective treatment after primary endpoints had been measured, patients and investigators were told whether the patient had been given placebo or active treatment after the result of the sting challenge was known and structured datasheets had been completed. Participants in the placebo group were offered VIT and the sting challenge was repeated after immunotherapy unless the patient had not reacted to their first sting challenge.

7.5 Sting challenge procedure

7.5.1 Clinical

To determine treatment efficacy, diagnostic sting challenges were performed in an emergency department resuscitation room 1 week after tolerating two successive VIT maintenance doses. Antihistamines were not taken for at least 1 week before the challenge. Patients were monitored non-invasively using electrocardiography, non-invasive blood pressure measurements, and continuous pulse-oximetry.

Table 7.3: Sting challenge anaphylaxis treatment guidelines

1.	**Oxygen** High flow oxygen (15 L/min) by facemask if SpO2 <92 or SBP<90 mmHg
2.	**Adrenaline infusion** 1 mg in 100 ml (1:100 000, 10 ug/ml) intravenously by infusion pump Commence at 30-100 ml/hr (5-17 ug/min) according to reaction severity – Titrate up or down according to response and side effects, aiming for lowest effective infusion rate – Tachycardia, tremor, and pallor in the setting of a normal or raised blood pressure are signs of adrenaline toxicity; consider a reduction in infusion rate Stop infusion 30 minutes after resolution of all symptoms & signs Continue observation for at least 2 hours after ceasing infusion (longer for severe or complicated reactions); discharge only if remains symptom-free
3.	**Normal saline rapid infusion** 1000 ml (pressurized) infused over 1-3 minutes and repeat as necessary Give if hypotension is severe or does not respond promptly to adrenaline
4.	**Hypotension resistant to above measures*** Consider bolus adrenaline, glucagon (5-10 mg IV bolus followed by infusion) and noradrenaline infusion with invasive blood pressure monitoring and central venous access.

* Planned contingencies, but not used during trial

With a 10 mL syringe device,[405] a single jack jumper ant was pushed against the ventral forearm and allowed to sting for 60 s. If no objective physical signs of a systemic reaction were evident within 30 min, a fresh ant was used to give a second sting. This second sting was given because during venom sac dissection approximately one in five venom sacs were empty.

Unless the trial participant wanted symptoms to be alleviated, reactions were allowed to progress until they were of stable severity for 30 min, or until peak expiratory flow rate fell to less than 60% of that at baseline, pulse oximetry saturation was under 92% breathing air, systolic blood pressure was less than 90 mm Hg, or if there was evidence of myocardial ischaemia. Treatment according to protocol (Table 7.3) focussed on oxygen, intravenous adrenaline infusion and volume resuscitation. Antihistamines and corticosteroids were not part of routine management because their usefulness for the management of the hyperacute phase of anaphylaxis was unknown and because theoretical considerations indicated they were unlikely to be beneficial. Discharge

home was permitted after a symptom-free interval of at least 2 hours following cessation of the adrenaline infusion (longer for severe or complicated reactions), or 1 hour after sting challenge if no reaction occurred.

For each reaction, the following data was recorded; interval from sting to onset of first symptoms, individual reaction features and events during treatment, whether symptoms recurred after a first attempt at ceasing the infusion, total dose and duration of the adrenaline infusion and other treatments administered. Differences between baseline physiological values and the highest heart rate, lowest systolic and mean blood pressures, lowest pulse oximetry oxygen saturation, lowest peak expiratory flow, and lowest forced expiratory volume/forced vital capacity after sting challenge were recorded. Reactions were graded in the same way as hypersensitivity reactions to VIT (Table 7.2).

7.5.2 Sting challenge blood sampling and laboratory analyses

Blood samples were taken prior to the sting (baseline), and 15 minutes and 60 minutes after the sting. Serum was collected from a clot-activator, serum-separating tube, kept at room temperature then frozen to minus 8 degrees Celsius within 2 hours. Plasma was collected from EDTA anticoagulated tubes that were stored on ice and spun down within 1 hour. The collection of plasma in this way was to prevent clotting-induced release of histamine from blood basophils.

Serum from each time point was sent to a national reference laboratory (John Hunter Hospital, Newcastle) for tryptase analysis (Unicap Tryptase, Pharmacia and Upjohn, manufacturers normal range <12 μg/L, detection limit 1 μg/L). Plasma samples from the baseline and 15-minute time points were analysed for histamine by ELISA (IBL Hamburg, manufacturers normal range <1.0 μg/L, detection limit 0.3 μg/L). Histamine determinations were limited to the 15-minute samples because previous studies have indicated this to be the optimum time for sampling histamine during sting challenges, with levels rapidly declining thereafter.[39,131,135]

7.6 In vitro laboratory studies

The following laboratory studies were performed in a laboratory set up specifically for the trial, with the assistance of three honours students (Matilda Haas, Andrew Black and Anand Parameswaran). Detailed methods, including reagents and a description of initial validation procedures and results for normal controls and a subset of patients enrolled in the early part of the trial, are reported in their respective theses.[406-408]

7.6.1 Blood sampling

Blood samples were taken at trial entry and prior to each sting challenge. For logistical reasons a full set of laboratory assessments could be performed at only one trial site (the Royal Hobart Hospital), where the majority of participants were enrolled, and some tests were unavailable temporarily during the trial.

7.6.2 RAST

Venom-specific IgE was measured by RAST [211], using venom bound to CNBr-activated discs. Results were expressed as percentages of anti-IgE tracer uptake. Serum was stored until completion of the clinical trial so that all samples from any one patient were analysed for IgE in a single laboratory assay. RAST studies at trial entry were performed at the NSL Health Research Laboratory. However, because NSL Health Ltd. ceased to provide these analyses during the trial we repeated the analyses in our own laboratory.

7.6.3 Basophil activation tests (BAT)

BAT were performed using the Basotest® kit (ORPEGEN Pharma) according to the manufacturer's instructions. Heparinised blood was primed with a stimulation buffer containing IL-3, incubated with negative control or positive control (fMLP) or venom for 20 minutes at 37 ºC, stained with anti-CD63-FITC and anti-IgE-PE, and analysed using a Coulter EPICS Elite ESP flow cytometer. The percentage of activated basophils was calculated by first gating the IgE positive granulocytes then determining the percentage of these cells expressing CD-63, defined by a marker set at the upper 2.5% of the negative control sample.

7.6.4 Histamine release tests (HRT)

HRT were performed on heparinised whole blood using histamine release kits and histamine ELISA (IBL Hamburg) according to the manufacturer's instructions. Heparinised blood was incubated with negative control, positive control (anti-IgE) or venom (without IL-3 priming) for 60 minutes at 37 ºC, or lysed to determine whole blood histamine content. Supernatants were stored at -20 ºC prior to analysis for histamine by ELISA. Results were expressed as the percentage of blood histamine released at each concentration of venom used.

7.6.5 Leukotriene release tests (LRT)

LRT were performed using the CAST-2000® kit (Bühlmann laboratories) according to the manufacturer's instructions. After sedimentation of erythrocytes with dextran,

leukocytes were separated by centrifugation, primed with IL-3, and incubated for 40 minutes at 37 ºC with negative control, positive control (anti-IgE) or venom. Supernatants were stored at -20 ºC prior to analysis by ELISA using a monoclonal antibody recognising LTC4, LTD4 and LTE4. Results were expressed in pg/mL.

7.6.6 Venom concentrations used for BAT, HRT and LRT

Venom concentrations of 0.001, 0.01 and 0.1 μg/ml were used for each test (BAT, HRT and LRT), as determined by a preliminary analysis for sensitivity and non-specific activation (which was evident at higher concentrations) in allergic volunteers and non-allergic controls.

7.6.7 Venom induced lymphocyte proliferation and cytokine excretion

Peripheral blood mononuclear cells (PMBC) were isolated from heparinised blood using a Lymphoprep™ gradient, washed with phosphate buffered saline and resuspended in RPMI-1640 medium containing 10% human AB+ serum and gentamicin, and adjusted to 2 x 10^6 cells/mL.

A total of 0.2 x 10^6 cells in 200uL were incubated for six days at 37˚C (in a fully humidified atmosphere of 5% $C0_2$ in air) with no stimulation (control), ConA at 12.5 ug/mL (positive control), or venom at 5 μg/mL, 1μg/mL, 0.5μg/mL, and 0.1μg/mL. Venom concentrations were chosen on the basis of a preliminary analysis for sensitivity and cytotoxicity thresholds in allergic and non-allergic allergic controls. 1μCi of ^{3}H-Thymidine was added to each well for the final 16 hours of culture. Proliferative responses were calculated as a stimulation indexes (SI), being the average CPM of triplicate antigen stimulated cultures divided by the average CPM of triplicate unstimulated cultures.

A total of 2 x 10^6 cells in 1 mL were incubated for six days at 37˚C (in a fully humidified atmosphere of 5% CO_2 in air) with no stimulation (control), PMA 1 μg/mL plus ionomycin 100 μg/mL (positive control), or *M. pilosula* venom at 1μg/mL (a concentration chosen as it was associated with the maximum response in proliferation studies). Supernatant samples were taken at day 6, and stored at -80˚C for later cytokine determinations by indirect sandwich ELISA (PharMingen), with results expressed in pg/mL. Initially we also analysed cytokine production at day 2, but after an interim analysis indicated more reliable detection at day 6 we stopped performing day 2 sampling. For PMA/ionomycin cultures, IL-4 and IFN-γ were analysed. Only IL-4 was analysed in venom culture supernatants, as IFN-γ was not found in detectable amounts either before or after VIT.

7.7 Analysis and power studies

7.7.1 Clinical immunotherapy trial

Power analysis showed that 40 people in each study group would give 85% power to detect a difference with a significance of α=0.05 with a two-tailed test, and assuming treatment failure rates of 30% (placebo) and 5% (active treatment). These were the average in-hospital sting challenge reaction rates in untreated individuals with a history of honeybee or wasp venom allergy and the average reported failure rate of VIT, respectively.[11,52,198]

The primary outcome measure was a systemic reaction to sting challenge defined by objective measurements. Secondary outcome measures were any systemic symptoms in the absence of objective physical signs, a grade IV reaction defined by hypotension, treatment with adrenaline, and changes in serum mast cell tryptase or plasma histamine after the sting challenge. An interim analysis was intended after the first 30 participants had completed the study. Fisher's exact and Chi-Squared tests were used to compare dichotomous and ordinal variables. The Mann-Whitney U test was used for continuous baseline variables, which had non-normal distributions. The median test was used to compare continuous outcome variables, all of which had non normal distributions that differed in shape between groups.

7.7.2 Sting anaphylaxis; clinical observations, management and diagnosis

Because VIT was expected to modifies reactivity, reducing the risk of severe anaphylaxis in this study, the analysis of clinical features was limited to reactions occurring in the placebo group. Relationships between clinical data elements and the total dose and duration of the adrenaline infusion were assessed with Analyse-it for Microsoft Excel,[409] using the Mann Whitney U test and Spearman rank correlation for continuous variables and the Chi-square test for categorical variables.

For the analysis of tryptase and histamine levels, results from all 64-sting challenges were included, to enable an adequate assessment of diagnostic performance. "Anaphylaxis" was defined by the occurrence of cardiorespiratory compromise corresponding with the major features of our grades III and IV (dyspnoea, wheeze, stridor, oxygen saturations <92%, or systolic blood pressure <90 mmHg).

For the highest tryptase level (peak-tryptase), sensitivity and specificity for a diagnosis of anaphylaxis were determined using the manufacturer's recommended cut-off, and an alternate cut-off according to receiver-operator characteristic (ROC) curve analysis. A ROC curve analysis was also used to determine an optimal diagnostic cut-off for

a change from baseline in tryptase (delta-tryptase) and this approach was compared with peak-tryptase by examining areas under the respective ROC curves. The same approach was used to determine the optimal use of plasma histamine, and then to test the utility of combining tryptase and histamine determinations. An assessment of variation in serum tryptase over time was made by comparing serum tryptase at trial enrolment with that at the time of sting challenge (baseline sample). Analyses were performed using Analyse-it for Microsoft Excel.[409]

7.7.3 Predicting sting challenge reactivity in the placebo group

For missing data, cases were excluded on a test-by-test basis. Significance was defined by a p value of <0.05. Baseline characteristics were compared between treatment groups using the Mann-Whitney U and Chi-squared tests for continuous and categorical variables respectively. The relationships between each baseline *in vitro* test and VST sensitivity threshold (1.0, 0.1 and 0.01 μg/mL in order of increasing sensitivity) were evaluated by Spearman rank correlation.

Analyses of *in vitro* test ability to predict the sting challenge result was confined to the placebo group, to avoid the confounding effect that VIT might have on laboratory parameters, and to match the real-life requirements of a test (i.e. to select untreated patients who would most benefit from VIT). The Pearson Chi-Square and Mann Whitney U tests were used to test for relationships between sting challenge result (occurrence of any grade reaction, or occurrence of a hypotensive reaction) and skin test sensitivity and *in vitro* tests respectively. A receiver-operator curve (ROC) analysis was performed for any test showing diagnostic potential, and 95% confidence intervals calculated for sensitivity and specificity.

To exclude patients from a therapy that may effectively prevent a life-threatening reaction to subsequent stings, a safe test for venom allergy would have to be ≥98% sensitive at the chosen diagnostic cut-off (that is, identify ≥98% of those who would react to sting challenge). A clinically useful test would have specificity in the order of 50% to enable exclusion of a significant proportion of people from needless treatment. Calculations using the computer program Power and Precision™ [290] indicated our study would have 83-89% power at a significance level of 0.05 to identify a clinically useful diagnostic test according to these requirements, if 30-50% of 30 people in the placebo treatment group reacted to sting challenge.

7.7.4 In vitro *parameter changes during VIT*

Changes in each *in vitro* parameter were assessed with the Wilcoxon signed-ranks test. Differences between placebo and venom groups were assessed with the Mann Whitney U test. Because relatively few sting challenge reactions were expected after VIT, this analysis was confined to identifying *in vitro* parameters that changed with VIT and thus might have potential for further investigation as markers of successful/ unsuccessful VIT.

7.8 Role of the funding sources

The sponsors of the study (NSL Health Ltd., Department of Health and Human Services Tasmania, Royal Hobart Research Foundation) had no role in study design, data collection, data analysis, data interpretation, or writing of the report. NSL Health Limited (Melbourne, Australia) discontinued allergy research activities and relinquished its commercial interests during the trial.

Chapter 8: Clinical trial of venom immunotherapy

8.1 Results

Between August 6, 2001, and Oct 4, 2001, 72 people were judged eligible for the trial on the basis of reaction histories, physical health, and willingness to undergo a sting challenge. Four were excluded because of negative skin tests- these people also had undetectable or equivocal serum IgE reactivity and did not have positive skin or serum IgE reactivity to honeybee, wasp, or venom from any other local *Myrmecia* spp. Thus, 68 individuals were randomly allocated to one of the two study groups– 33 to placebo and 35 to VIT (Figure 8.1). Table 8.1 shows participants' baseline characteristics. Table 8.2 shows main results of VIT, including adverse reactions.

After the first 30 participants completed sting challenges, a substantial imbalance between numbers in placebo and VIT groups was apparent (19 vs. 11, respectively). Interpretation of results from these 30 participants was difficult because taking a long time to achieve tolerance of the maintenance dose could well have been the result of treatment failure, and an interim analysis that excluded such cases would have been seriously biased. Therefore, the trial was continued.

In the placebo group, one participant departed overseas. Another withdrew because of newly diagnosed airways disease with deteriorating exercise tolerance. In the VIT group, one person developed debilitating panic attacks with flashbacks to a previous near-lethal reaction. Concealment of the patient's treatment was considered a contributing factor to their anxiety. After treatment allocation was revealed and psychiatric treatment was started, the panic attacks resolved and the patient continued with VIT.

After 52 sting challenges to participants for whom treatment allocation was not known, a severe grade IV reaction prompted another interim analysis. Of these challenges, objective reactions were noted in 21 of 29 (72%) in the placebo group and none of 23 in the VIT group ($p<0.0001$). Differences in the reaction rates would have remained significant even if the remaining two participants in the placebo group did not react and the remaining 12 participants in the VIT group did react to sting challenge ($p=0.0130$). Therefore, the placebo arm was terminated and all remaining treatment allocations were revealed.

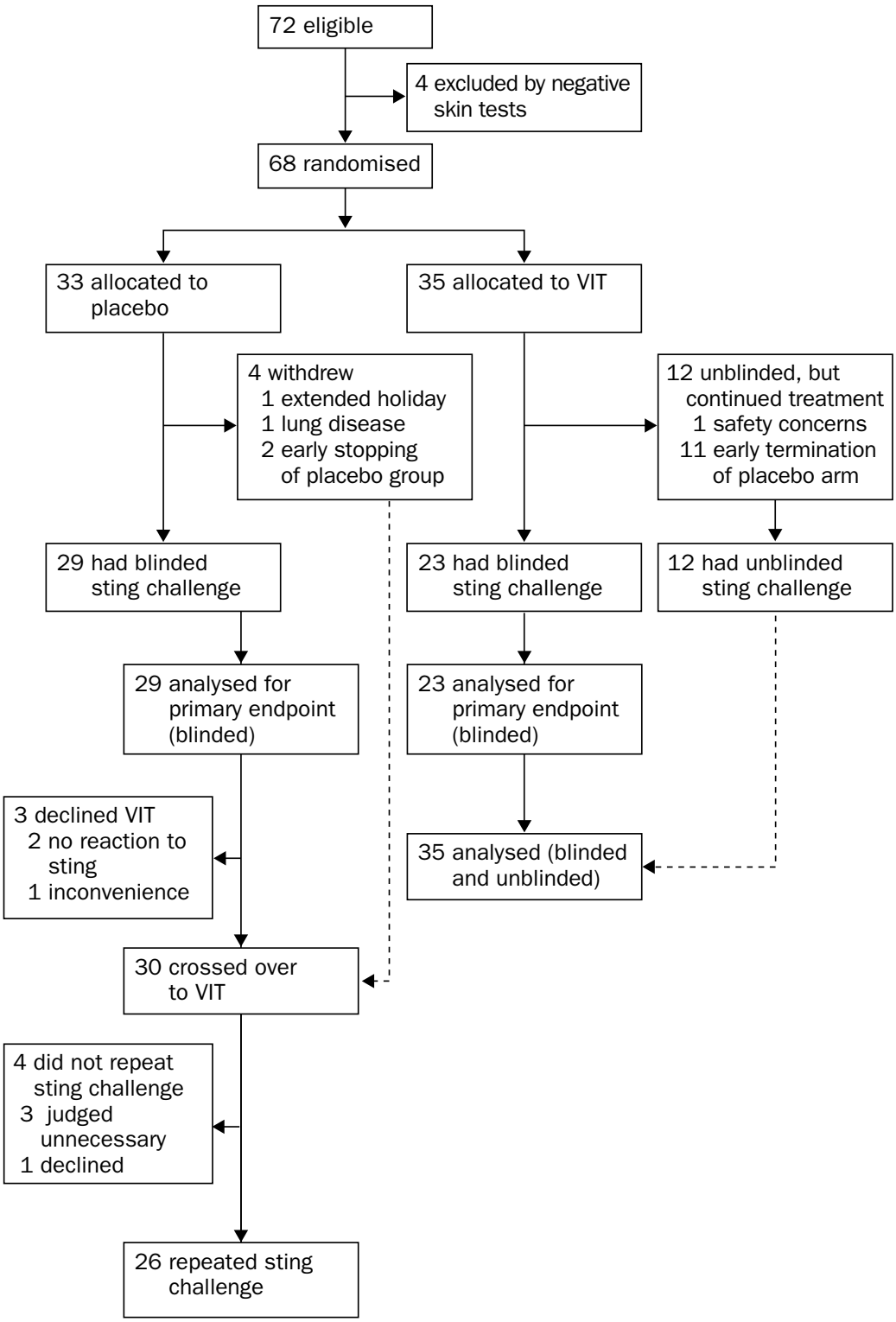


Figure 8.1: Trial profile

Table 8.1: Baseline characteristics of participants undergoing sting challenge

	Placebo (n=29)	**VIT (n=35)**	**p value**
Median age	46 (20-62)	50 (18-63)	0.1469
Male sex	18 (62%)	22 (63%)	0.8476
Asthma	0	5 (14%)	0.0852
M. pilosula clearly identified as causing a systemic reaction	29 (100%)	34 (97%)	0.9063
Worst previous reaction grade			0.1211
Grade II	1 (3%)	5 (14%)	
Grade III	20 (69%)	16 (46%)	
Grade IV	8 (28%)	14 (40%)	
Documented hypotension with sting	2 (7%)	9 (26%)	0.0925
Previous treatment with adrenaline	17 (59%)	29 (83%)	0.0616
Median age first systemic reaction	35 (4 – 60)	40 (9 – 49)	0.2404
Median years since last stung	3 (0 – 23)	2 (0 – 35)	0.4828
Median number of sting reactions	2 (1 – 20)	2 (1 – 24)	0.8618
Intradermal skin test positive threshold			0.6233
1.0 ug/ml	7 (24%)	5 (14%)	
0.1 ug/ml	11 (38%)	14 (40%)	
0.01 ug/ml or less	11 (38%)	16 (46%)	
Median venom-specific IgE *	1.9 (0.3 – 19)	3.8 (0.2 – 32.8)	0.3381
Baseline mast cell tryptase elevated	0	3 (9%) †	0.3142

Means and medians are presented with range.

*Venom specific serum IgE expressed as percentage uptake of radioactive tracer, as previously described [2].

† Tryptase levels (normal range <12 ug/L) were 16.3, 18.7 and 23.2 ug/L; all had a history of Grade IV anaphylaxis and one had historical features and skin findings suggestive of mastocytosis (telangiectasia macularis eruptiva perstans).

Table 8.2: Outcomes of randomised study phase

Immunotherapy	**Placebo (n=32*)**	**VIT (n=35)**	**p value**
Median number of visits †	11 (9 – 18)	15 (9 – 35)	0.003
Median cumulative dose (ug) †	568 (431 – 706)	648 (466 – 1971)	<0.001
H1/H2 blocker premedication	2	17	0.0002
Minor systemic symptoms	15	27	0.14
Number of people experiencing objective systemic reactions	1	13	0.0010
Treated with adrenaline	1	7	0.0064
Unable to reach 100 ug target dose	0	2	0.5548
Sting challenge	**Placebo (n=29)**	**VIT (n=35)**	**p value**
Any subjective symptoms (including tingling, transient itch, facial warmth, palpitations and headache)	26	13 ‡	<0.0001
Systemic reactions	21	1 ‡	<0.0001
Grade I	7	1‡	
Grade II	3	0	
Grade III	3	0	
Grade IV	8	0	0.0019
Treated with adrenaline	19	0	<0.0001
Median change tryptase (ug/ml)	1·4 (-3.7 – 31.3)	0·0 (-2.6 – 3.6)	<0.001
Median change histamine (ng/ml)	0·98 (-2.1 – 164)	0·0 (-4.1 – 3.5)	0.0287

Medians and means are presented with ranges.

*3 placebo cases withdrawn prior to sting challenge are included in the analysis of immunotherapy.

†Median number of visits and cumulative dose were calculated up to and including the 2nd maintenance dose. In people receiving VIT, 7 underwent at least 20 visits and received 1000-1971 ug of venom each. In 4 people, there were further delays to sting challenge ranging from 5-19 weeks, due to difficulties tolerating the 100 ug maintenance dose.

‡ Prior to early placebo arm termination and un-masking treatment allocations, the rate of systemic symptoms to sting challenge in the VIT group was 5/23 and objective reaction features 0/23.

In the placebo group, 15 anaphylactic reactions arose after the first sting and six after the second sting. Table 8.2 shows the severity of reactions to sting challenges. No participant requested treatment before the onset of severe reactions that were our endpoints. All responded well to treatment and made a full recovery. Sting reactions are described in more detail in Chapter 9:.

The severity of reaction to the deliberate sting in the placebo group did not seem to be predicted by the previous worst grade reaction to an accidental sting (Table 8.3), shown by five grade IV reactions that arose in the 20 people who had a history of grade III reactions.

After completion of sting challenges in the remainder of the VIT group, only one patient had a systemic reaction: a small patch of urticaria that resolved without treatment. This person had had a hypotensive reaction and needed resuscitation when last stung before trial entry. Maintenance doses of 100 μg had been tolerated without any side-effects before the sting challenge.

Thirty participants from the placebo group chose to receive VIT. Sting challenges were not repeated in three who did not have a reaction to sting challenge after placebo treatment and in one who had previously withdrawn from placebo treatment and declined a sting challenge after crossover (VIT). Of the remaining 26, only one reacted to sting challenge after crossover to VIT; this was a mild grade I urticarial reaction after one sting, that settled without treatment in a person who had a history of a grade IV sting reactions before trial entry, and who was unable to tolerate a maintenance

Table 8.3: Sting challenge reaction grade compared to prior worst reaction grade in the placebo group

Worst prior accidental sting reaction	Sting challenge reaction grade					
	Nil	I	II	III	IV	Total
Grade II	1					1
Grade III	5	4	3	3	5	20
Grade IV	2	3	0	0	3	8

Spearman rank correlation p value 0·99

Table 8.4: Adverse reactions to VIT; combined data from randomised and crossover phases.

Adverse reaction	Number affected (n=65)	Percentage (95% CI)
Minor systemic symptoms *	46	70.8% (58.2 – 81.4).
Immediate-type hypersensitivity reactions †	22	33.8% (22.6 – 46.6)
Worst grade I	16	
Worst grade II	1	
Worst grade III	3	
Worst grade IV	2	
Treated with adrenaline	10	12.3% (5.5 – 22.8)
Unable to tolerate maintenance due to repeated systemic reactions ‡	3	5% (1.0 – 12.9)

* Included generalised itch, vagueness, tingling of the lips or extremities, facial heat, tiredness/lethargy, chest pain, nausea, headache, abdominal pain, sensation of puffy eyes, perspiration and abnormal taste in the mouth.

† All three subjects with elevated baseline tryptase levels experienced hypersensitivity reactions during immunotherapy.

‡ Tolerated maintenance doses were 50-80 ug, despite antihistamine premedication. Only 1 of these 3 patients reacted (mild grade I, compared to previous grade IV reactivity) to sting challenge.

dose greater than 50 μg. Table 8.4 shows adverse reactions to VIT, including those in the crossover group.

8.2 Discussion

8.2.1 Principal findings

In this study, JJA VIT provided complete protection from sting anaphylaxis. Conversely, untreated participants were at substantial risk. For the time span investigated (3-6 months up to the time of a diagnostic sting challenge), immunotherapy was tolerated without any withdrawals despite a significant incidence of allergic hypersensitivity reactions to therapy.

8.2.2 Study strengths & weaknesses

The main strength of this study was its rigorous design and clearly defined patient group (otherwise healthy, skin test positive with a history of one or more severe JJA sting reactions).

Results in the placebo group indicated that the JJA sting challenge procedure was a useful test of treatment efficacy, able to precipitate severe anaphylaxis. The reaction risk in the placebo group was similar to the 70% (95% CI 61–78) risk from accidental field stings in JJA allergic people (see results 5.3.5) suggesting that the results of the in hospital sting challenges would be predictive of reactions to accidental stings.

Randomisation was effective, and groups were evenly matched in terms of characteristics known to be predictive of reaction severity- namely, age and severity of worst previous reaction.[52] With respect to blinding, clinically significant side-effects that distinguished people receiving VIT were unavoidable. Nevertheless, half the participants in the placebo group had systemic symptoms and many in the VIT group had few problems and progressed rapidly to maintenance dose early in the study before the placebo group was stopped. These results indicate that study blinding was sufficient to maintain objectivity.

8.2.3 Comparison with related studies

8.2.3.1 Adverse reactions to VIT

Hypersensitivity reactions during VIT occurred in all three patients who had raised baseline concentrations of tryptase, which lends support to mast cell disease as an important factor in some people with severe allergy.[202] The proportion of participants who had hypersensitivity reactions and failed to achieve the target maintenance dose were much the same as those reported for outpatient honeybee VIT, but greater than for wasp VIT.[209]

8.2.3.2 VIT Efficacy

The finding here that VIT is an efficacious treatment is consistent with previous studies of honeybee and wasp sting allergy (see literature review, 1.5.1.2). However, the combination of a very high reaction rate in untreated patients, high systemic reaction rate to VIT, and virtually 100% efficacy is interesting. Experience with other insect allergies suggest that high reaction rates to sting challenge and VIT are associated with high failure rates after VIT. Honeybee VIT is associated with high reaction rates in untreated people to sting challenge, high systemic reaction rates during therapy and a noticeable failure rate (in the order of 10-20% as measured by deliberate sting

challenge). In contrast, untreated vespid wasp allergic people have low reaction rates to sting challenge, VIT is better tolerated and VIT failure rate is close to 0%.[283]

The low failure rate with JJA VIT despite what is apparently a highly allergenic sting may be due to the high maintenance dose to sting dose ratio. JJA venom sacs each contain ~30 μg dried weight of venom compared to honeybee venom sacs which contain 100-300 μg venom. Nevertheless, 100 μg maintenance doses have been used for both. Experience with honeybee venom allergy suggests that a lower maintenance dose is associated with higher failure rates,[272] and that treatment failures at the standard 100 μg maintenance dose can be overcome using higher doses of venom extract.[201] Conversely, JJA VIT might remain highly efficacious in most people using a lower maintenance dose.

8.2.3.3 Sting challenge rates in the placebo group

These sting challenge reaction rates seen in the placebo group of this study and during the prospective study of JJA field stings (see results 5.3.5) were much higher than the 27–57% rates reported in large prospective studies of untreated people with honeybee and wasp sting allergies incorporating deliberate sting challenges.[52,198,245,398-400] It is not known whether this high rate is because JJA venom is an unusually potent allergen, because of high yearly exposure rates in Tasmania that might maintain sensitivity in allergic patients (Chapter 5:), or because of the ability of JJA to reliably deliver venom by virtue of its strong grasp and sting mechanism.

It is noteworthy that several people with a history of grade III reactions had grade IV reactions after the sting challenge. Although not identified in one series of sting challenges,[52] progression of reaction severity has been documented previously for honeybee and wasp sting allergy,[198] and was noted in the results presented in Chapter 5:.Outside a critical care environment, hypotension might not be detected because it can be a transient event associated with largely subjective symptoms.[40] Thus, some reactions will probably be graded as more severe when closely monitored. Also, since severity of sting reactions is known to fluctuate over time, initial reactions might be minor and subsequent reactions more severe.

Superficially, this finding contrasts with that of Chapter 5:, where prior worst reaction severity was a good predictor of subsequent reaction severity. However, this clinical trial focused on those with severe allergy. The epidemiological study included people with mild allergy and was more able to identify risk differences between those with a history of severe allergy and those with a history of mild allergy.

8.2.4 Interpretation

In this highly motivated, highly allergic, but otherwise healthy study population who had positive intradermal skin tests, VIT was very effective in prevention of life-threatening sting anaphylaxis. VIT has some potential to prevent deaths, and can also result in striking improvements in the quality of life of affected individuals.[410] The high systemic reaction rate in the placebo group of this study indicates that the use of a diagnostic sting challenge to determine suitability for immunotherapy (as suggested for people with honeybee and wasp allergy) is unnecessary, indeed dangerous, in those with a clear history of severe allergy to JJA venom and a positive skin test.

Ant VIT could benefit about 1% of the population (those with severe allergy) in areas of southeastern Australia where *Myrmecia* ant stings occur and should be offered to those with skin test venom reactivity and a history of grade III–IV reactions. As with VIT for other sting allergies, the risk of systemic reactions to JJA venom means that treatment should be given where there is immediate access to resuscitation facilities.

8.2.5 Unanswered questions

How VIT could benefit the small proportion of skin-test negative patients is still unclear, as such patients were excluded from this study. Four excluded people had clearly reacted to JJA stings in the past but were skin test negative, with no skin or serum sIgE reactivity to any local insect species. This dilemma also applies to other hymenoptera sting allergies.[107]

As this study did not include people with mild reactions, further deliberate sting challenge data in such people would be useful to confirm that they are at low risk of a severe reaction. This is especially important given the findings here that progression to a more severe reaction can occur. Diagnostic challenges in people who have previously experienced mild reactions might be useful to identify those at risk of having a more severe reaction to subsequent stings.

Real-life application of VIT in less motivated patients than those in this study might result in poor outcomes if patients withdraw because of adverse effects of treatment.[209] In this context, the minimum effective dose in JJA VIT and long term efficacy and tolerability remain to be defined. Real-life monitoring of VIT administered outside the idealised clinical trial environment, including people with comorbidities and including children, is required.

Chapter 9: Sting anaphylaxis; manifestations, management and diagnosis

9.1 Results

9.1.1 Clinical description of reactions in the placebo group

There were 7 grade I, 3 grade II, 3 grade III and 8 grade IV reactions in the placebo group. Main clinical features of the 11 severe reactions to sting challenges are described in Table 9.1 and the 10 mild reactions are presented in Table 9.2.

The time interval between sting and symptom onset ranged from 2-27 (median 8) minutes. The most common first symptoms were generalised itch and abnormal perioral sensations (tingling lips or tongue, or abnormal taste) in 10 and 7 people respectively. Erythema (+/- urticaria) was the initial physical sign in all cases apart from case 5 (see below).

Skin features, although frequently subtle, were identified in all (generalised erythema 100%, itch 82%, urticaria 68%). Angioedema occurred in 7(33%), colicky abdominal pain (including severe "period-like" pains in one person) occurred in 4 (19%) and respiratory features (dyspnoea, or wheeze) occurred in 7(33%). One grade III and three grade IV reactions were accompanied by pulse oximetry saturations of 92% or less. Lowest measured systolic and mean pressures in grade IV reactions ranged from 0-88 (median 71) mmHg and 0-55 (median 45) mmHg respectively. Hypotensive reactions were characterised by an initial fall in diastolic blood pressure, indicating systemic vasodilation, and all were accompanied by an initial tachycardia followed by relative bradycardia with a heart rate drop of 15-65 (median 32) beats per minute accompanying the onset of hypotension. ST segment abnormalities occurred in two reactions (cases 5 and 12, see below).

Adrenaline infusions were given for 19 reactions, including all those of grade II-IV severity. The two remaining urticarial reactions resolved without treatment. Five of the eight people with hypotension were also given a 1L saline bolus during the first few minutes of resuscitation. All responded rapidly to treatment, with symptomatic improvement and systolic blood pressure rising above 90 mmHg within 5 minutes except one who continued to deteriorate and was given a further 2L of saline (case 3, see below). Two were given atropine for bradycardia (cases 3 and 5, see below). There were no appreciable adverse reactions to treatment.

Table 9.1: Details of severe (grade III-IV) sting challenge reactions

Case number		Onset *	Reaction features
Grade IV (hypotensive) reactions	1	5 minutes	Gen. urticaria, angioedema, chest tightness, hypotension (MBP 41)
	2	2 minutes	Gen. urticaria, angioedema, dysphagia, hypotension (MBP 55), hypoxaemia (SpO2 93%)
	3	5 minutes	Gen. erythema, severe bradycardia given atropine, hypotension (MBP 42) resistant to fluids and adrenaline
	4	5 minutes	Gen. urticaria, dyspnoea, cough, wheeze, hypotension (MBP 54), hypoxaemia (SpO2 89%)
	5	20 minutes	Gen. erythema, dysphagia, dyspnoea, severe bradycardia given atropine, hypotension (MBP unrecordable), unconsciousness
	6	18 minutes	Gen. erythema, angioedema, dyspnoea, wheeze, hypotension (MBP 48), hypoxaemia (SpO2 88%)
	7	5 minutes	Gen. urticaria, severe abdominal pain, dyspnoea, hypotension (MBP 52)
	8	10 minutes	Gen. urticaria, hypotension (MBP 41), prolonged adrenaline infusion (recurrent urticaria on several attempts at ceasing infusion)
Grade III reactions	9	12 minutes	Gen. urticaria, abdominal pain, dyspnoea, mild wheeze
	10	8 minutes	Gen. erythema, angioedema, chest tightness, cough, wheeze, hypoxaemia (SpO2 92%)
	11	6 minutes	Gen erythema, abdominal pain, cough, wheeze

* Onset = time from sting to first symptoms. MBP = Mean Blood Pressure. Urticaria = classical generalised erythema + wheal type skin eruption. Erythema = generalised skin erythema.

The median total dose of adrenaline was 590 ug (range 190-1310 ug) and median total infusion duration was 115 minutes (range 52-292 minutes). Total adrenaline doses and infusion durations are plotted against reaction grade in Figure 9.1. Hypotensive reactions received significantly more adrenaline (median 762ug vs. 520 ug, p 0.02) and longer infusions (median 169 vs. 92 minutes, p 0.03). Adrenaline dose and infusion duration did not correlate significantly with any other clinical parameter. In 9 patients, 7 of whom had grade III-IV reactions, the first attempt at ceasing the infusion was followed by a reaction recurrence and the infusion recommenced. This was after a median initial infusion time of 67 minutes in the group with symptom recurrence, versus 79 minutes in those without recurrence, a non-significant difference. Corticosteroids and antihistamines were prescribed for three people; one with mild urticaria that kept recurring when the adrenaline was stopped, and two with very large local reactions at the sting site the following day.

Table 9.2: Details of mild (grade I-II) sting challenge reactions

Case number		Onset *	Reaction features
Grade II reactions	12	8 minutes	Gen. erythema, angioedema, chest tightness, dysphagia, ST segment depression and T wave inversion†
	13	12 minutes	Gen. urticaria, angioedema, chest tightness
	14	19 minutes	Gen. erythema, malaise, angioedema, abdominal pain, dysphagia,
Grade I reaction	15	13 minutes	Gen. urticaria
	16	6 minutes	Gen. urticaria
	17	4 minutes	Gen. urticaria
	18	5 minutes	Gen. urticaria, chest tightness,
	19	8 minutes	Gen. urticaria
	20	8 minutes	Gen. erythema
	21	27 minutes	Gen. urticaria
	22	5 minutes	Gen. urticaria, dizziness,

* Onset = time from sting to first symptoms. MBP = Mean Blood Pressure. Urticaria = classical generalised erythema + wheal type skin eruption. Erythema = generalised skin erythema. † according to the grading system prospectively designed for use in this research this reaction is classed as grade II; however according to another similar grading system used in sting challenge studies, it would be designated as grade IV because of apparent cardiac involvement (see Table 1.9).

Case 1 (Figure 9.2A) illustrates a typical hypotensive reaction. A mild increase in heart rate follows the initial sting and then settles. The reaction begins with tachycardia and a fall in diastolic and mean blood pressures, indicating peripheral vasodilation. Systolic hypotension and a slowing of the heart rate follow this.

Case 3 (Figure 9.2B) was characterised by sudden visual loss and throbbing severe headache followed by hypotension. Despite rapid infusion of 2L saline over 5 minutes and adrenaline infused at 30 ug/min, progressive bradycardia required treatment with atropine 600 ug intravenously. At the same time, extravasation around the intravenous cannula was noted, where infusions had inadvertently been set on the same side that minutely blood pressure estimations were being performed. After swapping infusions to the back-up cannula there was gradual improvement over the following 5-10 minutes. Notably, previous reactions had been characterised by visual loss and breathlessness without any other symptoms suggesting hypotension. These reactions responded promptly to intramuscular adrenaline.

Case 5 (Figure 9.2C) began with a sensation of a “lump in the throat”, followed within 3 minutes by unconsciousness, agonal respirations, and absent pulses. Atropine,

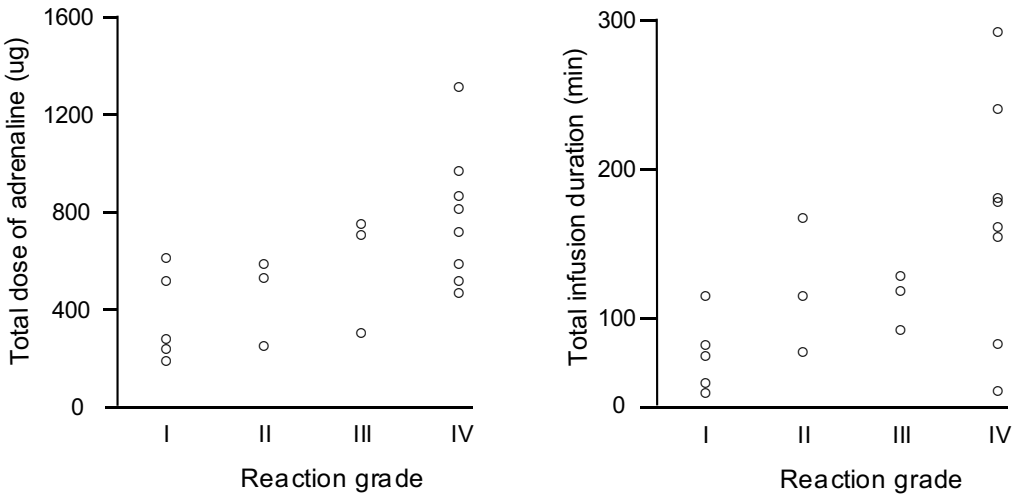


Figure 9.1: Total dose of adrenaline and total infusion duration by reaction grade

adrenaline, and saline infusions were effective. Generalised erythema developed shortly after. One hour later an attempt was made to cease the adrenaline infusion but florid erythema developed accompanied by a fall in blood pressure. Inferior T wave inversion was noted on her ECG during the reaction; this resolved without any CK or troponin rises, and an outpatient exercise sestamibi scan was unremarkable.

Case 12 was a grade II reaction characterised by urticaria, angioedema and chest tightness. There was no fall in either systolic or diastolic blood pressure, and heart rate did not rise above 110 bpm. ECG developed marked T wave inversion and mild ST depression (Figure 9.3) prompting treatment with adrenaline. Although the ST depression improved with treatment, T wave inversion took several weeks resolve. Serial CK and troponin were normal as was an outpatient exercise sestamibi scan.

9.1.2 Serum tryptase and plasma histamine

Tryptase and histamine levels for participants experiencing systemic reactions are presented in Figure 9.4 (mild reactions) and Figure 9.5 (severe reactions), and can be compared with the clinical features presented in Table 9.1 and Table 9.2 respectively. A ROC curve analysis for tryptase measurements is presented in Figure 9.6. Using the manufacturer's normal range cut-off (12.0 ug/L) for peak-tryptase, sensitivity and specificity were 0.36 (95% CI 0.11-0.69) and 0.89 (95% CI 0.77-0.96) respectively. Using a cut-off of 9.0 ug/L derived from the ROC analysis, sensitivity appeared to improve (0.55, 95% CI 0.23-80.3) with no significant loss of specificity (0.87, 95% CI

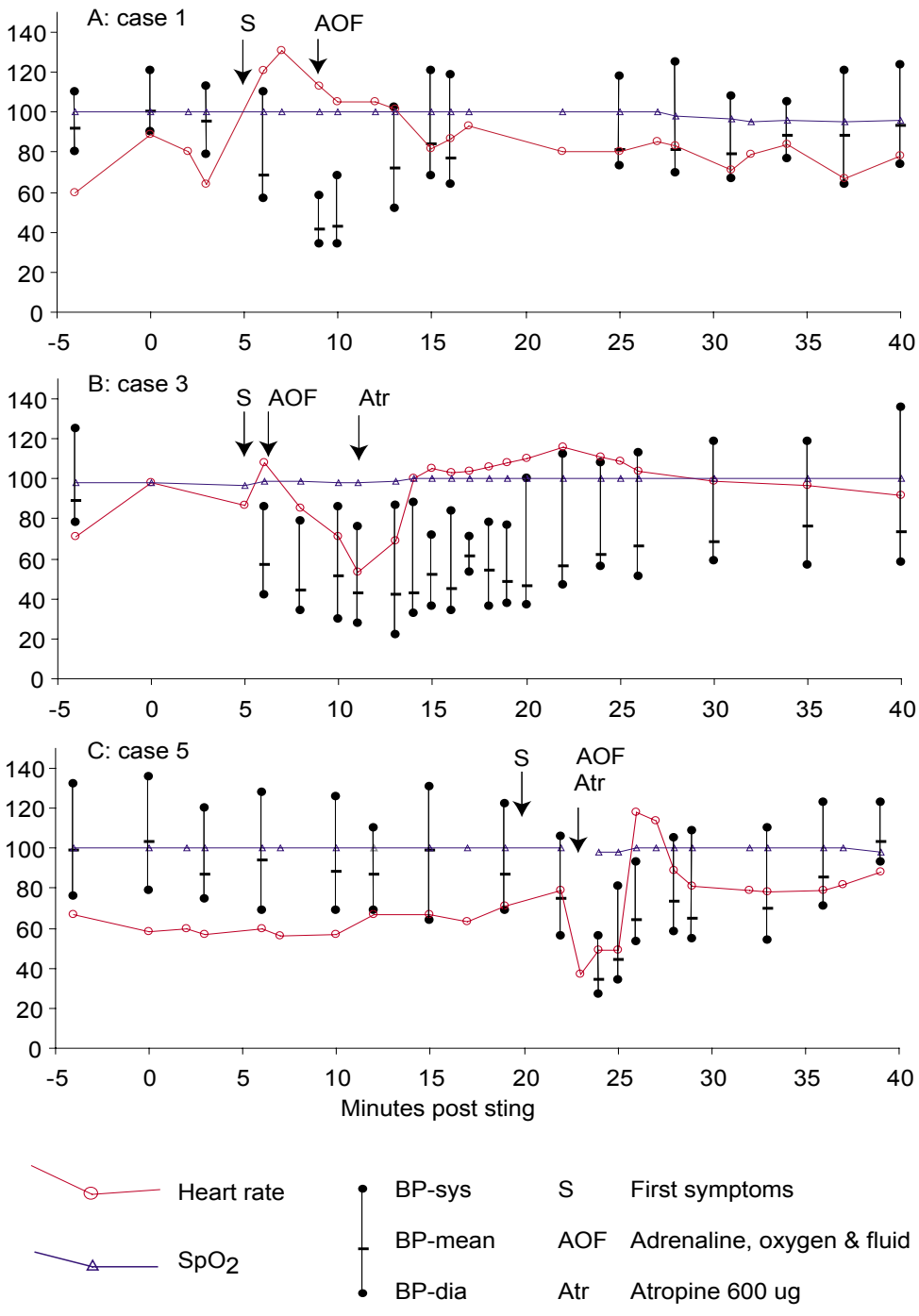


Figure 9.2: A-C: Physiological observations and treatment, cases 1, 3 and 5

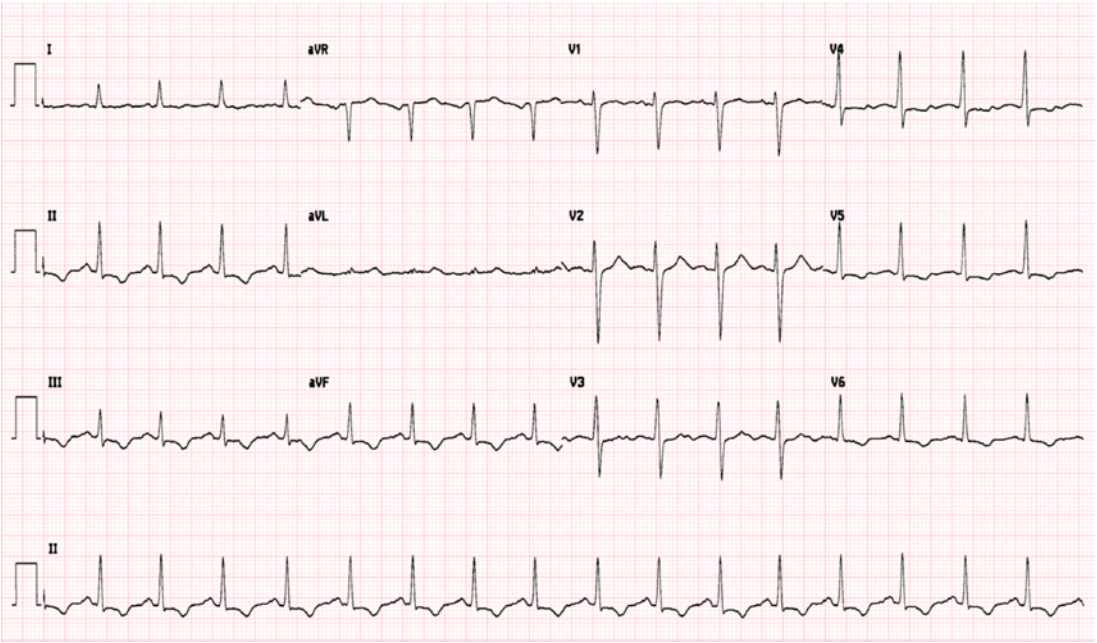
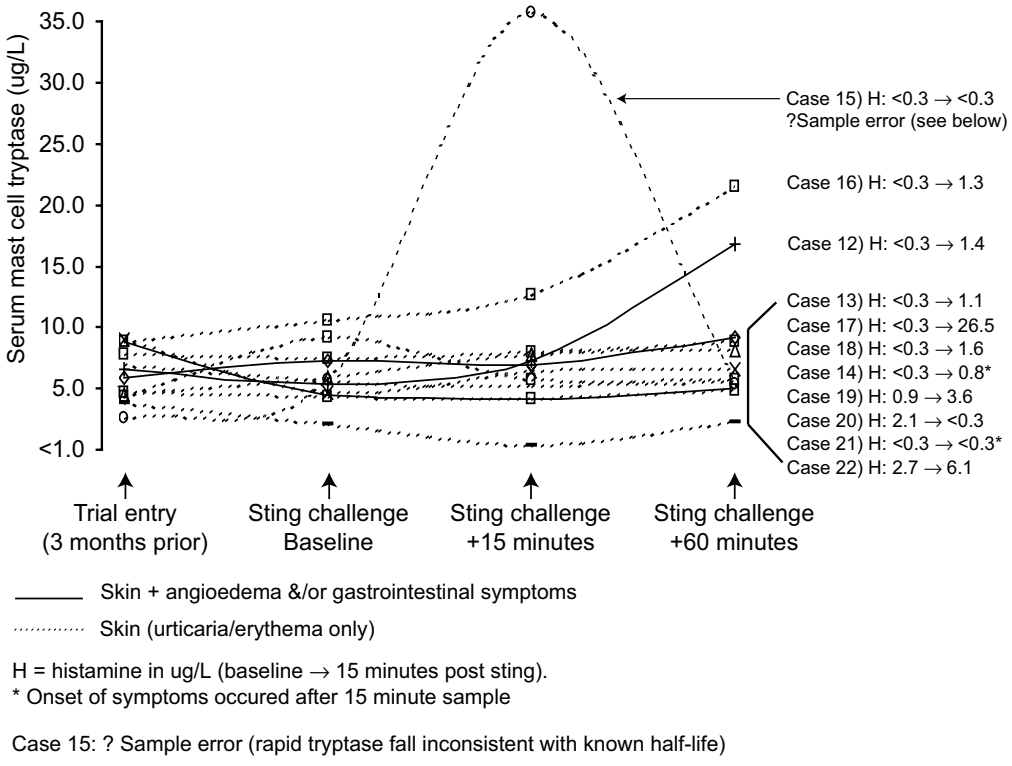


Figure 9.3: Case 12- electrocardiogram prior to commencing adrenaline infusion

0.75-0.95). Delta-tryptase performed significantly better than peak-tryptase (p = 0.0172 for the areas under the ROC curves being equal). A delta-tryptase of 2.0 ug/L gave a sensitivity of 0.73 (95% CI 0.39-0.94) and specificity 0.91 (95% CI 0.79-0.97).

Peak-histamine measurements using the manufacturers cut-off (>1.0 ug/L) had sensitivity 0.70 (95% CI 0.35-0.93) and specificity 0.69 (95% CI 0.54-0.81). ROC curve analysis found that a cut-off of 2.0 ug/L was optimal, with sensitivity 0.60 (95% CI 0.26-0.88) and specificity 0.88 (95% CI 0.76-0.96). Delta-histamine of >2.0 ug/L had sensitivity 0.60 (95% CI 0.26-0.88) and specificity 0.94 (95% CI 0.84-0.99).

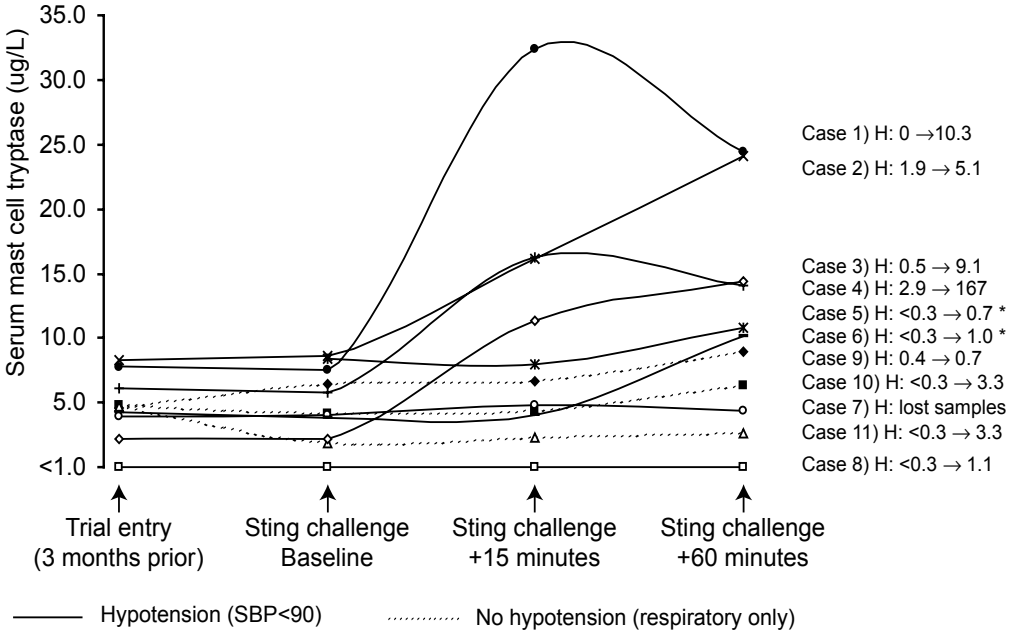
Mild reactions, allocated to the non-anaphylaxis group according to our clinical definition, were responsible for four false positives in each of the peak and delta-tryptase analyses, six false positives in the peak-histamine analysis and three false positives in the delta-histamine analysis. Elevated baseline tryptase levels (all >16.0 ug/L) were responsible for another three false positives in the peak-tryptase (but not delta-tryptase) analyses. If mild reactions were excluded from the analysis, sensitivities were unchanged but specificities increased to 0.93 (95% CI 0.81-0.99) for peak-tryptase, 0.98 (95%CI 0.87-1.0) for delta-tryptase, 0.93 (95% CI 0.80-0.98) for peak histamine, and 1.0 (95% CI 0.91-1.0) for delta-histamine.



H = histamine in ug/L (baseline → 15 minutes post sting).
* Onset of symptoms occured after 15 minute sample

Case 15: ? Sample error (rapid tryptase fall inconsistent with known half-life)

Figure 9.4: Tryptase and histamine changes in mild (grade I-II) reactions



H = histamine in ug/L (baseline → 15 minutes post sting).
* Onset of symptoms occured after 15 minute sample

Figure 9.5: Tryptase and histamine changes in severe (grade III-IV) reactions

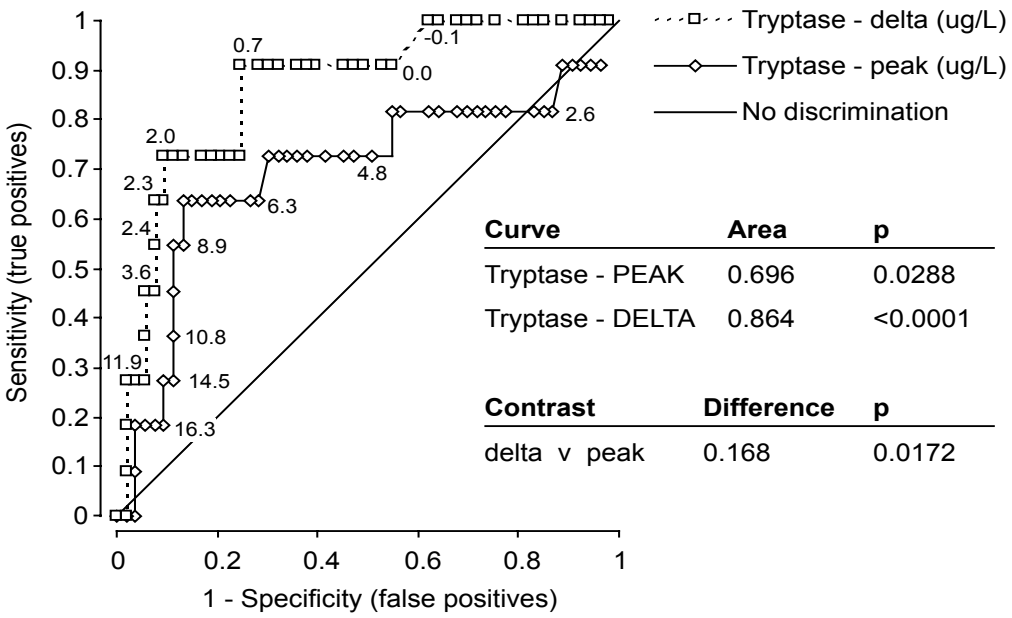


Figure 9.6: ROC curve analysis for tryptase measurements, comparing use of peak tryptase measurement versus delta-tryptase calculation (see Figure 5.2 for an explaination of ROC methodology)

Maximum sensitivity and specificity, 0.90 (95% CI 0.56-1.0) and 0.84 (95% CI 0.71-0.93) respectively, were obtained using a combination of either a delta-tryptase >2.0 ug/L or delta-histamine >2.0 ug/L, with specificity increasing to 0.98 (95% CI 0.87-1.0) when mild reactions were excluded from the analysis.

Over the 14-week period prior to sting challenge, the mean and median changes in tryptase levels were 0.26 and 0.30 respectively (range –5.0 to 5.4 ug/L, interquartile range 1.3 ug/L).

9.2 Discussion

9.2.1 Principal findings

There was a consistent and rapid clinical improvement after commencing treatment according to protocol. The efficacy of adrenaline in particular was evident in the recurrence of reaction features on stopping the infusion, resolving rapidly again with its re-institution in 9 cases. The fluid volumes given were less than reported from reactions occurring during anaesthesia,[45] which may be a reflection of differences in antigen load and reaction severity, or blunted responsiveness due to anaesthetic

agents. No major adverse reactions to treatment were identified, although in one case inadvertent infusion on the same side as frequent NIBP measurements probably delayed response to treatment and caused a degree of drug and fluid extravasation.

Significantly more adrenaline was used to treat hypotensive than non-hypotensive reactions. Relative bradycardia always accompanied hypotension and in two cases was treated with intravenous atropine; one appeared to be progressing towards cardiac arrest and the other had no detectable pulses. In one case the myocardium was clearly involved in the anaphylactic process without any evidence of circulatory instability, although it was impossible to determine whether this involvement was secondary to coronary vasospasm or an effect of anaphylactic mediators on the myocardium. One reaction also suggested the nervous system as a target organ for anaphylaxis, with visual loss occurring prior to cardiovascular compromise.

Peak-tryptase measurements lacked adequate sensitivity to exclude a diagnosis of anaphylaxis in this series. False positives using peak-tryptase also occurred due to people with elevated baseline levels. Measuring change in tryptase from baseline significantly improved both sensitivity and specificity, as tryptase levels appear to be stable in the absence of an allergic reaction. The addition of histamine determinations may increase diagnostic sensitivity, however the confidence intervals of this study are wide and the results inconclusive in this regard.

9.2.2 Study strengths & weaknesses

A gold standard for the diagnosis of anaphylaxis has not been defined, although a position statement by the European Academy of Allergology and Clinical Immunology (EAACI) defines anaphylaxis as “severe, life-threatening, multiple-organ hypersensitivity, often dominated by severe asthma and hypotension”, but also stating “these (asthma and hypotension) do not have to be present for a reaction to be classified as anaphylaxis”.[105] Clearly, half of the reactions seen here did not satisfy this definition. A strength of the study is that when assessing the diagnostic sensitivity of tryptase and histamine measurements, a definition was used that included unequivocal signs of systemic mediator release plus respiratory &/or cardiovascular compromise.

Other strengths of this study are that reaction features and response to treatment were monitored in a controlled environment according to a consistent protocol, a situation rarely possible when studying human anaphylaxis due to its infrequent and emergent nature. Weaknesses include the exclusion of patients with co-morbidities and absence of a control (untreated) group, however these limitations were essential for patient safety. Also, the small number of cases prevented a confident assessment of how the

various clinical parameters might predict the amount of adrenaline required, and gave relatively wide confidence intervals when assessing the diagnostic characteristics of tryptase and histamine. This also made it impossible to perform confident subgroup analyses to determine whether tryptase rises were more likely in hypotensive reactions than predominantly respiratory reactions, or whether the magnitude of rise was inversely related to the degree of hypotension, as found in one previous study of experimentally-induced sting anaphylaxis.[131]

9.2.3 Comparison with related studies

9.2.3.1 Clinical issues

Human data on the efficacy and safety of pharmacological treatments for anaphylaxis is limited. Management guidelines, which emphasise a central role for adrenaline,[139-141] are based largely on expert opinion. Reactions can spontaneously resolve with endogenous compensatory responses,[40] but failure to use adrenaline has been considered a major factor contributing to lethal outcomes.[54,67] However clinical observations of severe anaphylaxis in humans,[39] as well as canine experiments,[146] suggest that a single dose of adrenaline may produce only transient improvement.

The apparent success of the treatment protocol using adrenaline and volume resuscitation is consistent with findings by Fisher, who observed rapid improvement with adrenaline as well as evidence of fluid extravasation of up to 35% of circulating blood volume within 10 minutes of reaction onset.[45] The rapid response of patients to our treatment protocol contrasts with a report of 17 patients with anaphylactic shock deliberately induced by insect sting and treated only with fluids and antihistamines, where “all but two recovered within 4 hours”.[40]

Protracted anaphylaxis requiring large doses of adrenaline and noradrenaline following deliberate sting challenge has been reported.[39] Health-based exclusion criteria do not appear to have been applied in that study, where a large antigen load was also administered by graded subcutaneous injection prior to the sting- possibly contributing to the severity of the subsequent reactions. However, adrenaline-resistant anaphylaxis has been recognised as an important albeit rare phenomenon probably associated with impaired cardiac function. Patients have been successfully resuscitated in this situation with large doses of adrenaline and noradrenaline,[39,45] amrinone,[152] glucagon,[151] and mechanical (intra-aortic balloon pump) support.[41] The observation of marked ECG changes in a reaction without cardiovascular compromise or subsequent evidence of coronary disease is consistent with the increasing recognition of the human heart,

which contains significant numbers of mast cells,[95] as a major shock organ in some cases of anaphylaxis.[81,89]

Relative bradycardia (falling heart rate despite worsening hypotension) has been reported previously in the setting of deliberately induced sting anaphylaxis,[39,40] but may be under-recognised clinically where a rapid demise occurs prior to reaching medical care. For example, a recent sting death observed by us was characterised by sudden collapse with a severe bradycardia manifested as a slow idioventricular rhythm noted 5 minutes later on the arrival of paramedics; at the time we attributed this finding to hypoxia (Chapter 3, Case 4).[395394]

Possible explanations for the bradycardia identified here include an effect of anaphylactic mediators on the heart &/or nervous system, and neurocardiogenic mechanisms. Bradycardia may be a non-specific feature of severe hypovolaemic-distributive shock. Physiological studies of awake mammals have identified two phases of response to hypovolaemia, an initial phase of blood pressure maintenance by tachycardia and peripheral vasoconstriction, followed by a second phase with more severe hypovolaemia that is characterised by bradycardia, reduced peripheral vascular tone and a profound fall in blood pressure.[411]

The mechanisms involved may be similar to those implicated in neurocardiogenic syncope. In that condition, excessive activation of the cardiac mechanoreceptors by mechanical stimulation (increased force of contraction) and chemical factors during a period of sympathetic excitation is thought to combine with potentiated central reflexes to trigger both a parasympathetic outflow and a dramatic reduction in sympathetic nerve outflow.[412] Catecholamines and prostaglandins appear to sensitise cardiac mechanoreceptors, while serotonin (5-hydroxytryptamine) and nitric oxide have been found to potentiate the central reflexes.[413] Levels of these mediators are known to be elevated during anaphylaxis.[40,131,414] Thus, during anaphylaxis neurocardiogenic mechanisms may both exacerbate peripheral vasodilation and cause bradycardia. This may be lethal when combined with other features of anaphylactic shock. Bradycardia has not been reported as a major feature of anaphylaxis under anaesthesia,[45] perhaps related to the blunting of central reflexes in that setting, or because such physiological changes are less likely to be identified in retrospective studies.

9.2.3.2 Tryptase and histamine measurements

Unlike these results, one previous study of sting challenges found tryptase elevations to be a constant finding in severe sting anaphylaxis, although a ROC curve analysis was not presented.[131] Another study of severe adverse reactions occurring under anaesthesia

found peak-tryptase to be a useful marker of anaphylaxis using an older Pharmacia RIA system and lower tryptase cut-offs (3-5 ug/L).[388] Interpretation of sensitivity and specificity in that study was hampered by lack of a diagnostic gold standard; the detection of specific IgE was used as a proxy marker of anaphylaxis. Specific IgE was detected in 125/158 with raised tryptase and 14/154 without raised tryptase. A difficulty encountered analysing these and other previous studies lies with differing methodologies of tryptase estimation. Older methodologies including the Pharmacia RIA measure mature tryptase (elevated only with mast cell degranulation), whereas the contemporary Pharmacia UniCAP system measures both mature and precursor forms of tryptase that are released spontaneously in the absence of anaphylaxis.[415]

A study of post-mortem tryptase including 10 anaphylactic deaths found sensitivity of 0.86 and specificity of 0.88 using a 10 ug/L cut-off.[392] Another found tryptase to be elevated in 14/16 anaphylactic deaths (sensitivity 0.88).[156] Although limited in the number of cases studied, these studies suggest that tryptase may be more reliably elevated in lethal reactions than for non-lethal reactions. However, tryptase may not enter the circulation until 30 minutes after exposure, peaking 1-2 hours later if the circulation remains intact.[390] In sting deaths the median time to cardiac arrest is only 15 minutes.[67,99] Therefore, where death rapidly supervenes tryptase levels may not have the opportunity to rise. In the small series of JJA sting anaphylaxis deaths reported in Chapter 3:, tryptase levels were markedly elevated in only 1 of 3 cases where these levels were measured with borderline elevations above the normal range in the other two.[395]

In a study of emergency department presentations with generally mild allergic reactions and using a relatively high diagnostic cut-off (13.5 ug/mL), measurement of tryptase had a sensitivity of 20/97 (0.2) for the diagnosis of acute allergic syndromes (including mild reactions). Adding histamine measurements increased diagnostic sensitivity, however the difficulty ensuring plasma samples were handled with the necessary care in a real-life clinical environment was noted.[134] Another issue with using histamine measurements is one of timing, given that the early peak during anaphylaxis can be easily missed.[39,131,135]

9.2.4 Interpretation

9.2.4.1 Clinical issues

This study supports the use of anaphylaxis treatment protocols that incorporate oxygen, intravenous adrenaline infusions and volume resuscitation, and clinical observations suggest a supplementary role for atropine in cases associated with severe bradycardia.

The large doses of adrenaline required for hypotensive reactions also raises concerns that the standard Epipen dose (0.3 mg) for self-administration may be inadequate for people experiencing a severe reaction.

Extrapolation to other forms of anaphylaxis and to patients with comorbidities should be done with caution. There is some evidence that anaphylaxis to ingested antigens is more likely to involve severe bronchospasm,[67] and delayed phase reactions,[53] where additional bronchodilator treatment, corticosteroids, and prolonged periods of observation may be required. Practitioners also need to be aware of the phenomenon of adrenaline-resistant anaphylaxis and consider additional treatment measures including more aggressive volume resuscitation, higher doses of adrenaline, noradrenaline, glucagon, amrinone and balloon-pump support. Furthermore, this study was performed under the supervision of emergency medicine specialists in a well-equipped resuscitation room. The treatment protocol may not be applicable to other clinical settings.

9.2.4.2 Diagnosis

Measurement of tryptase levels, whilst not useful in acute management, may facilitate subsequent assessments and management planning, by confirming the diagnosis where there has been any clinical doubt. However, caution is required. During anaphylaxis, an elevation of tryptase within the reference range is common. In the absence of anaphylaxis, tryptase remains stable and does not vary by more than 2.0 ug/L in any given individual over a short timeframe. Anaphylaxis-induced elevation of tryptase, as well as false positives, may only be recognised if serial measurements are performed.

9.2.5 Unanswered questions

The utility of steroids and antihistamines for managing anaphylaxis is unknown, as are the relative benefits and risks of using intravenous infusions of adrenaline in preference to intramuscular adrenaline. The treatment model examined here could be used to investigate the usefulness of steroids and antihistamines, using the recurrence of symptoms when first ceasing the adrenaline infusion as an endpoint. A direct comparison of intravenous versus intramuscular adrenaline may also be warranted.

The real-life utility of tryptase +/-histamine assays in general emergency medicine practice where patients present after the onset of anaphylaxis is unknown. Further “real life” studies of diagnostic performance and clinical utility are required.

Chapter 10: Predicting sting challenge reaction risk

10.1 Results

10.1.1 Correlations between laboratory parameters and skin testing

Spearman correlation coefficient values for the relationship between venom-specific laboratory parameters at each venom concentration at trial entry and VST threshold are outlined in Table 10.1. As can be seen, the HRT was the only venom-specific laboratory parameter that did not correlate significantly with VST. For BAT and LRT studies, the best correlation with VST sensitivity appeared to be at a venom concentration of 0.01 ug/mL.

10.1.2 Predicting sting challenge reactions in the placebo group

A cross-tabulation of VST sensitivity and sting challenge reactivity in terms of both any reaction occurrence and severe (hypotensive) reactivity is presented in Table 10.2. Participants sensitive at a venom concentration or 0.01 ug/mL were significantly more likely to experience a hypotensive reaction. A cross-tabulation of *in vitro* results at trial entry and sting challenge reactivity is presented in Table 10.3. Although there were generally higher median values for many tests in those experiencing systemic reactions, these differences were not statistically significant. Only the HRT differed significantly between non-reactors and reactors, at a venom concentration of 0.1 ug/mL. Using a venom concentration of 0.01 ug/mL, HRT values in those with hypotensive reactions were significantly higher than those with no or mild reactions.

ROC curve analyses for the HRT are presented with sensitivity, specificity, and positive and negative predictive values in Figure 10.1. In the clinical context of this study, a high sensitivity was required to detect potentially life-threatening allergy. Therefore, high sensitivity/moderate-low specificity points on the ROC curve were chosen as the optimal diagnostic thresholds. To screen for any degree of sting challenge reactivity, an HRT of >10% at a venom concentration of 0.1 ug/mL was optimal (sensitivity 94%, specificity 50%). Using the same diagnostic cut off, the test was 100% sensitive for severe reactivity (that is, any degree of respiratory compromise or hypotension). To screen for hypotensive reactions, an HRT of >6.5% at a venom concentration of 0.01 ug/mL was optimal (sensitivity 100%, specificity 39%). However, as noted in Figure 1, the confidence intervals were wide.

Table 10.1: Correlation between venom-specific laboratory parameters and venom skin test sensitivity threshold

	Venom concentration (ug/mL) of *in-vitro* test						
	0.001	**0.01**	**0.1**	**0.5**	**1.0**	**5.0**	**N/A**
sIgE							rs=0.44 p<0.001
SI			rs=0.37 p=0.193	rs=0.37 p=0.004	rs=0.40 p=0.002	rs=0.34 p=0.008	
IL-4							
HRT	rs=0.07 p=0.608	rs=0.25 p=0.085	rs=0.17 p=0.248				
BAT	rs=0.39 p=0.003	rs=0.55 p<0.001	rs=0.46 p<0.001				
LRT	rs=0.31 p=0.019	rs=0.40 p=0.002	rs=0.29 p=0.026				

rs = Spearman correlation coefficient.

Table 10.2: Venom skin test sensitivity versus sting challenge sensitivity

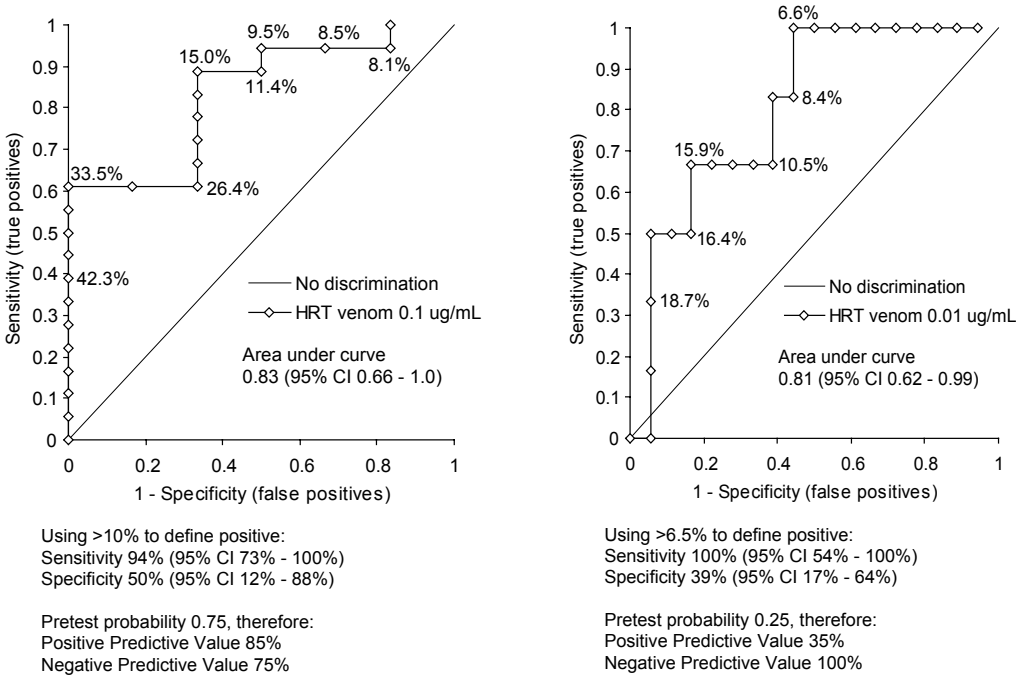
		Total	**No reaction**	**Objective systemic reaction**	
				Non-hypotensive	**Hypotensive**
Skin test sensitivity, ug/mL*	0.01	11	1 (9%)	4 (36%)	6 (55%)
	0.1	11	4 (36%)	6 (55%)	1 (9%)
	1.0	7	3 (43%)	3 (43%)	1 (14%)
p value (Chi square)			Any reaction: 0.210	Hypotensive reaction: 0.039	

* Venom concentration at which skin test noted to be positive; lower concentrations equal greater sensitivity.

Table 10.3: *In vitro* test results versus sting challenge sensitivity

		No reaction	Systemic reaction		Hypotensive reaction	
	n	Median (Range)	Median (Range)	p	Median (Range)	p
sIgE RAST (% tracer uptake)						
	29	2.1% (1-27)	6.5% (1-35)	0.153	7.2% (3-35)	0.126
Lymphocyte SI						
0.1	29	1.7 (0.5-25.5)	2.1 (0.4-28.4)	0.981	2.9 (1.5-28.4)	0.083
0.5	29	6.8 (1.9-37.6)	4.6 (0.7-53.2)	0.582	7.0 (3.2-33.4)	0.153
1.0	29	8.9 (2.9-45.0)	6.0 (1.1-47.7)	0.401	9.1 (4.2-35.1)	0.103
5.0	29	10.6 (2.3-36.8)	9.4 (1.0-82.5)	0.615	14.9 (3.2-48.7)	0.349
Lymphocyte IL-4 production (pg/mL)						
Venom	29	9.5 (0-109)	11.0 (0-425)	0.981	43 (0-425)	0.114
PMA	29	121 (0-2990)	288 (0-3880)	0.237	80 (5-950)	0.649
Lymphocyte IFN-γ production (pg/mL)						
PMA	29	1540 (0-8210)	1605 (0-19410)	0.793	1652 (0-19410)	0.867
HRT (% of blood histamine released)						
0.001	24	2.2% (0-7)	1.7% (0-15)	0.820	2.4% (0-6)	0.923
0.01	24	5.5% (0-18)	11.9% (2-62)	0.280	17.6% (8-21)	0.027
0.1	24	12.2% (0-33)	39.2% (8-67)	0.015	39.8% (18-67)	0.177
BAT (% of basophils activated)						
0.001	26	5.4% (2-9)	5.1% (1-41)	0.778	6.4% (1-41)	0.494
0.01	26	34% (3-53)	43% (7-82)	0.135	31% (7-82)	0.790
0.1	26	36% (6-75)	64% (7-87)	0.152	62% (7-85)	0.836
LRT (pg/mL)						
0.001	26	70 (0-287)	96 (0-715)	0.866	117 (0-715)	0.421
0.01	26	187 (97-1585)	343 (31-1100)	0.364	493 (82-1100)	0.295
0.1	26	326 (102-6554)	481 (0-3807)	0.120	593 (308-1041)	0.457

Venom concentrations for the test are recorded in the left-hand column in μg/mL. Significant differences are highlighted in bold. The p values in this cross-tabulation are for comparisons of test values between those with no reaction and those with any grade of systemic reaction, and between those with no or mild reactions and those with hypotensive reactions.



Using >10% to define positive:
Sensitivity 94% (95% CI 73% - 100%)
Specificity 50% (95% CI 12% - 88%)

Pretest probability 0.75, therefore:
Positive Predictive Value 85%
Negative Predictive Value 75%

A: Screening for any grade of reaction to sting challenge (HRT at a venom concentration of 0.1 ug/mL)

Using >6.5% to define positive:
Sensitivity 100% (95% CI 54% - 100%)
Specificity 39% (95% CI 17% - 64%)

Pretest probability 0.25, therefore:
Positive Predictive Value 35%
Negative Predictive Value 100%

B: Screening for hypotensive reaction to sting challenge (HRT at a venom concentration of 0.01 ug/mL)

Figure 10.1: ROC curve analysis for the histamine release test (see Figure 5.2 for an explaination of ROC methodology)

10.2 Discussion

10.2.1 Principal findings

The results of venom-specific IgE RAST, SI, IL-4 production in response to venom, LRT, and BAT all mirrored VST sensitivity. However, only VST and HRT appeared to be predictive of sting challenge results. With a higher concentration of venom (0.1 ug/mL), release of >10% of total blood histamine was a reasonably sensitive marker of any degree of sting challenge sensitivity, and 100% sensitive for severe reactivity. Using a lower venom concentration of 0.01 ug/mL, release of >6.5% of total blood histamine was less sensitive for any degree of reactivity, but was 100% sensitive for hypotensive anaphylaxis. However, because of small sample size, the confidence intervals for these estimates were wide.

10.2.2 Study strengths & weaknesses

The main strengths of this study were its randomised placebo-controlled design and use of a diagnostic sting challenge (incorporating two stings) as the gold standard for determining treatment efficacy. The study was also performed in a clinically relevant

group of patients, namely those who are currently considered as candidates for immunotherapy, and used commercially available quality-controlled test kits.

Limitation of this study were that:

(i) Although diagnostic sting challenge is the best available test for venom allergy, it is not infallible because a small proportion of people (approximately 5%) with initially negative challenges may go on to react to subsequent field stings;[204]

(ii) The subgroup with hypotensive anaphylaxis was relatively small (necessary for ethical reasons);

(iii) For logistical reasons tests could not always be performed;

(iv) A simple approach in assessing IL-4 release was employed, namely venom stimulation of cell cultures. Methods that select venom-reactive cells prior to non-specific stimulation may be more useful.[313]

(v) The concentration of venom for lymphocyte cytokine studies was based on highest proliferative responses. In this context, it is notable that one of the major allergenic peptides in *M pilosula* venom (pilosulin 1) is cytotoxic and may have influenced our results.[375] The possibility exists that tests using different venom concentrations, sampled at different time points of the lymphocyte culture, or using pre-selected venom-reactive cell lines followed by non-specific stimulation, could have performed better.

10.2.3 Comparison with related studies

The findings here are consistent with previous studies that have shown BAT and LRT tests to correlate well, but HRT to correspond less well, with VST in the setting of honeybee and yellow jacket venom allergy.[234,235] However, they are not consistent with one large sting challenge study performed in the setting of honeybee and vespid allergy that found VST did not predict sting challenge sensitivity.[52] Whether this inconsistency is due to chance, differences in VST methodology, or differences between honeybee, wasp and *M pilosula* allergy is unknown. Significant differences in natural history,[52,198] diagnostic performance of skin tests and serum IgE analysis,[219] and behaviour during VIT[283] have been observed in comparisons of honeybee and vespid wasp allergy. *M pilosula* allergy appears to be unique in terms of a high reaction rate to both accidental

and deliberate stings when compared to honeybee and vespid allergy, so differences in diagnostic test performance can also be anticipated.

An explanation for the finding that HRT (the only test not correlating with skin test sensitivity) predicted sensitivity whereas BAT and HRT did not may lie with the use of IL-3 pre-incubation in the BAT and LRT (but not HRT) tests. One previous study showing some correlation between HRT and VST utilised an IL-3 pre incubation step during the HRT.[235] Alternatively, other factor(s) having an effect on the links between basophil activation and mediator release may be important.

10.2.4 Interpretation

Further research into the utility of the HRT is warranted. Although specificity appears to be poor, the inconvenience and cost of VIT means that even the exclusion of a small number of people who would otherwise be given VIT might make the test cost-effective.

10.2.5 Unanswered questions

A larger study is required to confirm the sensitivity (with narrower confidence intervals) of HRT in identifying those at risk of JJA sting anaphylaxis. Studies including other insect allergies will also be required, as the discriminating concentrations using insect venoms in not known. A larger definitive study of diagnostic sensitivity must include prospective follow up of accidental stings and perhaps repeated sting challenges at a later date to identify those in whom allergic sensitivity re-develops after the initial sting challenge. It remains to be determined whether the HRT would be useful in determining reaction risk in people with negative VST- a group currently not offered immunotherapy but who may still be at a significant risk.[107]

Chapter 11: Changes in laboratory parameters with VIT

11.1 Results

All baseline laboratory parameters were compared between placebo and VIT groups to detect any randomisation bias not evident in the analysis of clinical characteristics. There were no significant between-group differences (data not shown) except for the HRT at a venom concentration of 0.01 ug/ml, where the median histamine releases were 7.5% and 2.3% in the placebo and venom groups respectively (p =0.029).

Box plots and significance values for laboratory parameter changes demonstrating significant changes during immunotherapy are presented in Figures 11.1 to .

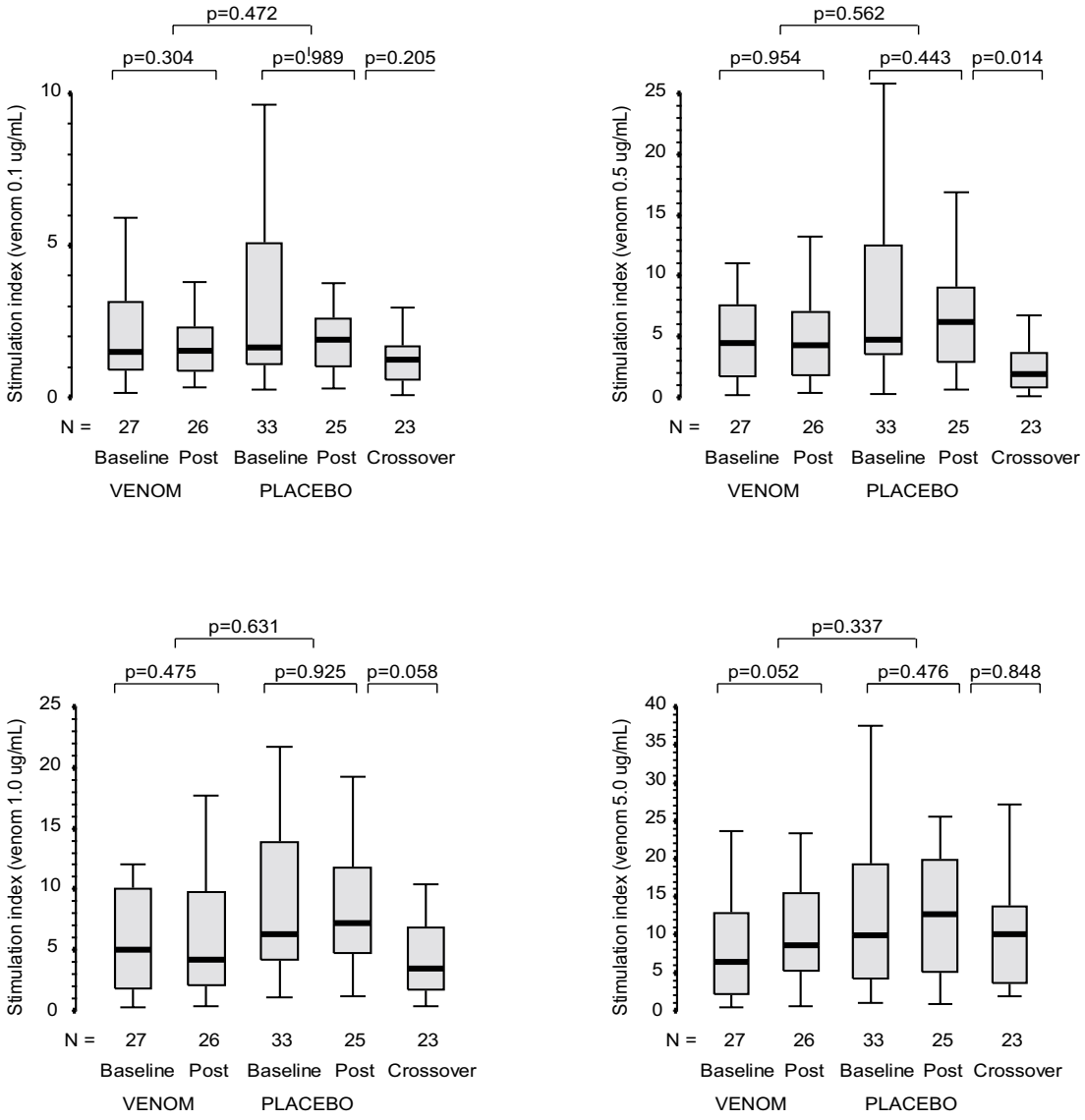
These may be summarised as follows:

(i) Venom-specific IgE (RAST) remained unchanged during placebo treatment, but significantly increased with immunotherapy during both the randomised and crossover phases.

(ii) Reductions in the HRT, BAT and LRT were evident during placebo treatment. In comparison, during VIT (randomised and crossover), there was either an increase or a lesser decline. However, for the randomised phase statistically significant differences between venom and placebo groups were only evident with the HRT where median histamine release increased after VIT but fell with placebo treatment.

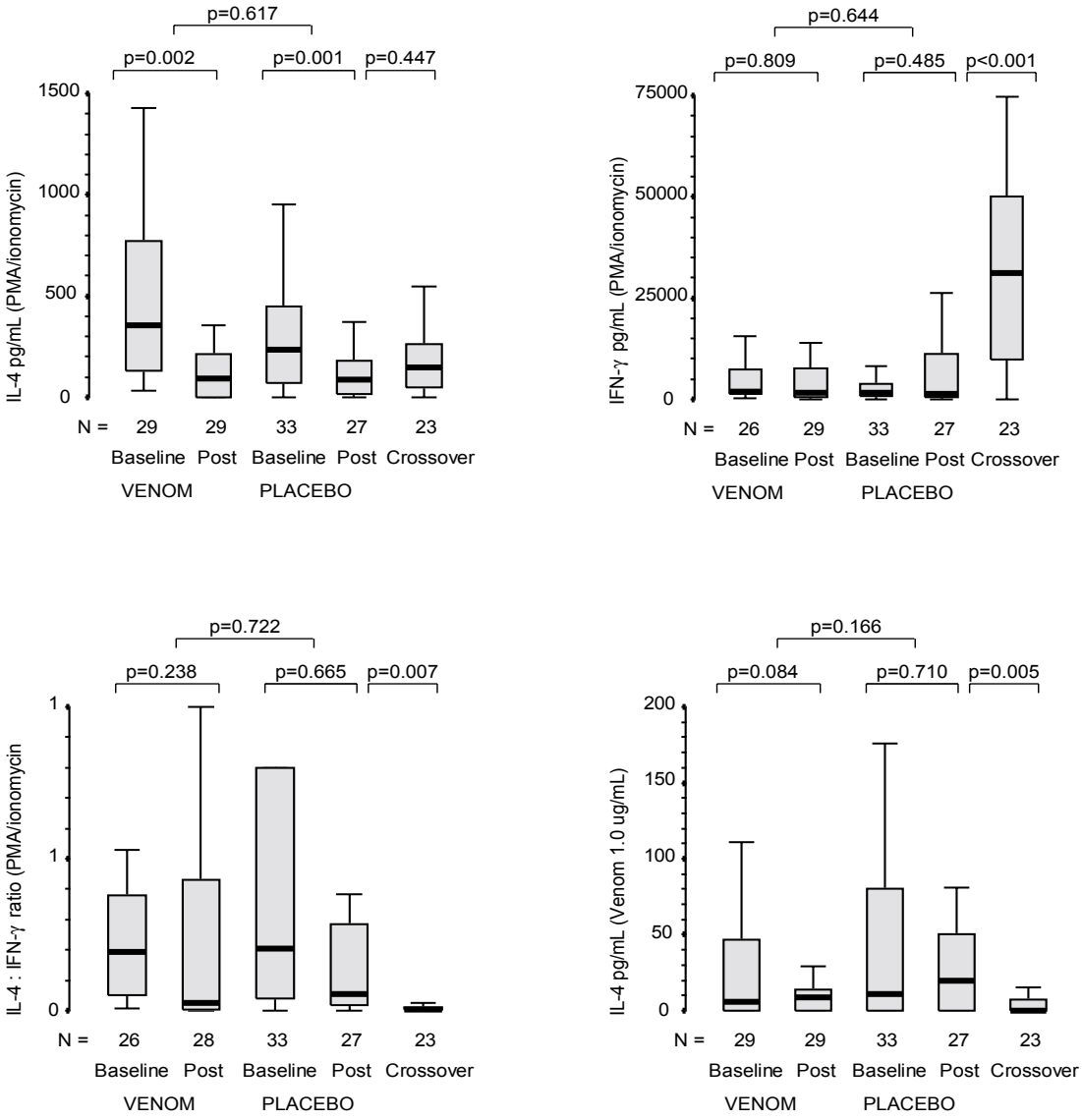
(iii) Lymphocyte proliferative responses to venom did not differ significantly between venom and placebo groups. However, lymphocyte IL-4 production in response to venom appeared to be reduced with VIT, with a reduction in the number of "outliers" with very high IL-4 production in both groups undergoing VIT, statistically significant in the crossover group.

(iv) IL-4 production in response to non-specific stimulation with PMA and ionomycin fell after treatment in both venom and placebo groups during the randomised phase, but did not change during the crossover phase. Conversely, IFN-γ production increased during crossover treatment, but no change was seen during the randomised phase.

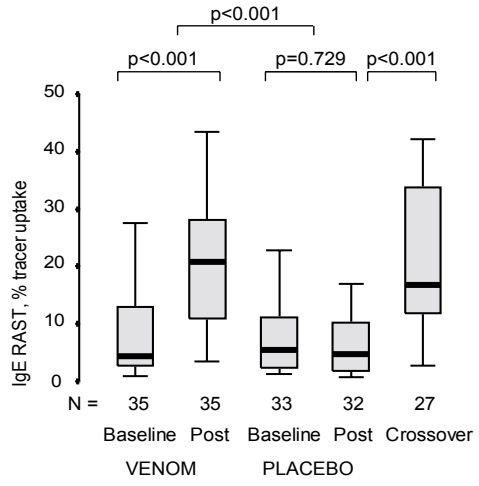


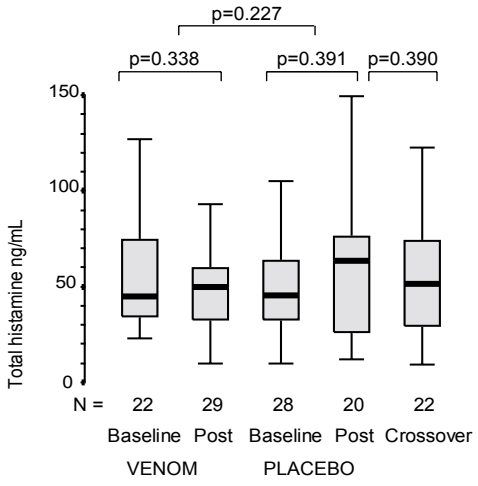
11.1A: Lymphocyte proliferation (stimulation index).

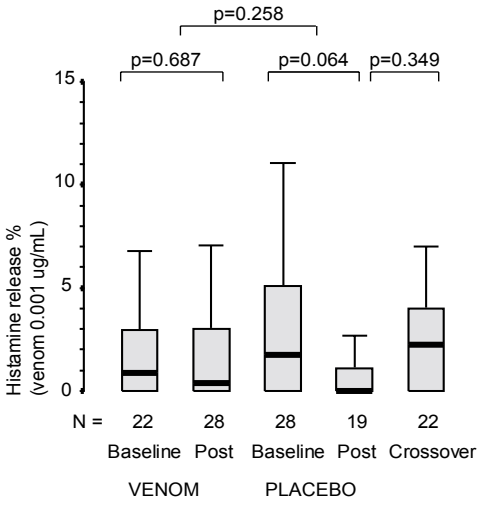
Figures 11.1 A-D: *In vitro* changes during immunotherapy: The three lower p values for each graph are for changes between pre- and post- treatment values for each treatment arm (Wilcoxon signed-ranks test). The top p value compares changes in the parameter between placebo and venom groups during the randomised phase of the trial (Mann Whitney U test).

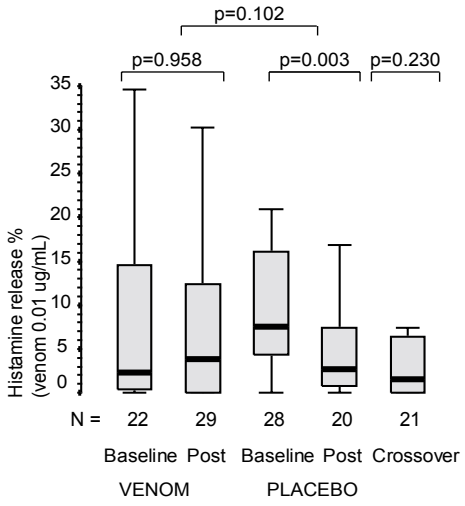


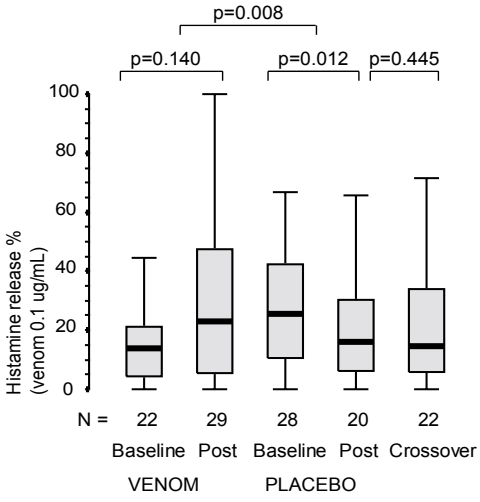
11.1B: IL-4 and IFN-γ



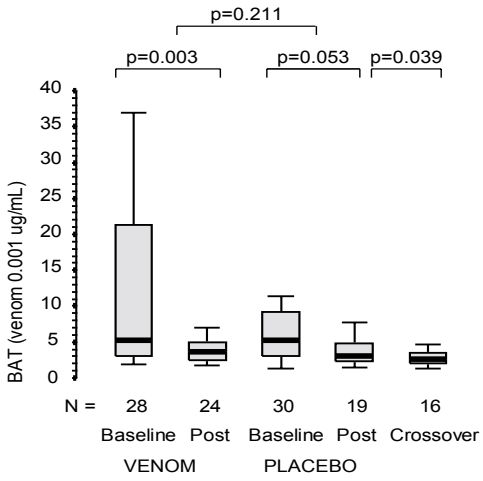


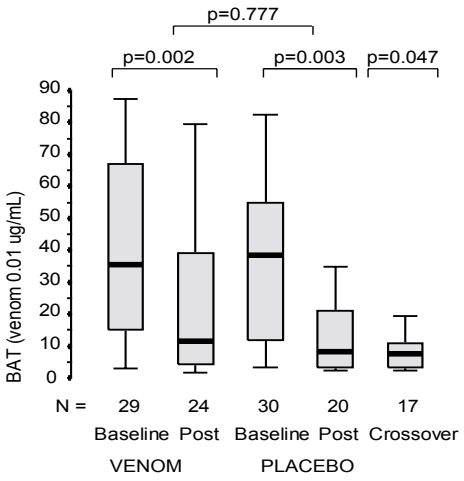


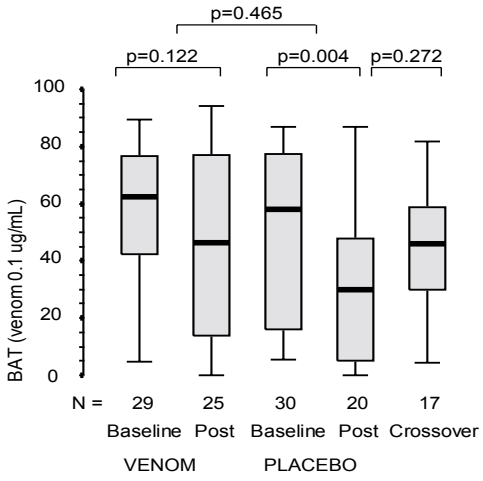


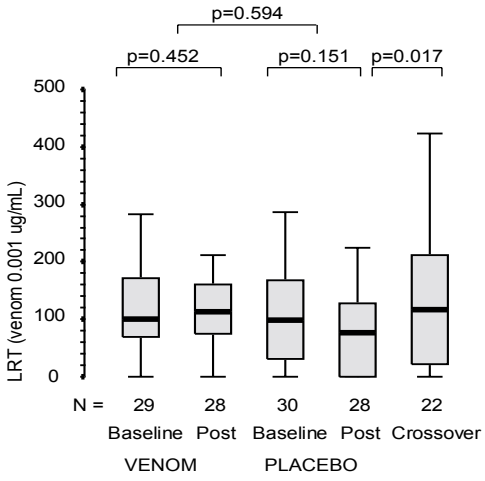


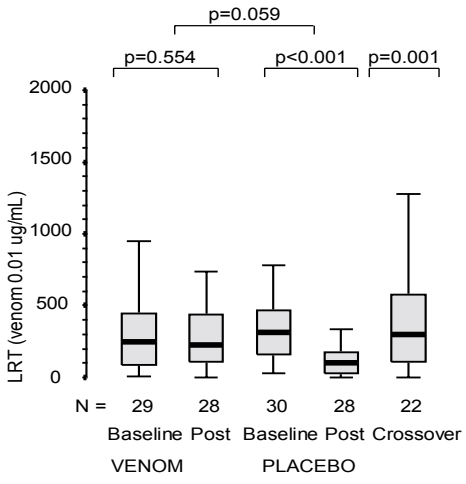
11.1C: IgE RAST & Histamine release test (HRT)

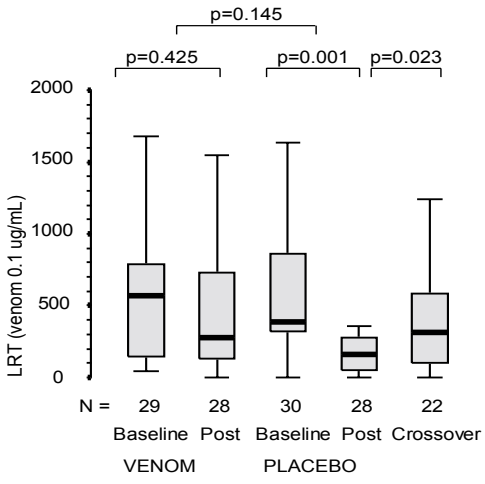












11.1D: Basophil activation test (BAT) & Leukotriene release test (LRT)

11.2 Discussion

11.2.1 Principal findings

During the trial, changes in the various laboratory parameters appeared to be somewhat erratic. When directly comparing the placebo and VIT groups, the only statistically significant changes found with VIT were an increase in venom-specific IgE RAST and a reversal of the trend towards reduced HRT values seen in the placebo group. It appeared that VIT may prevent a "natural" decline in BAT and LRT reactivity seen in the placebo group. Although an effect of VIT on leukocyte IL-4 production was evident taking together the changes seen in both randomised and crossover phases, this did not reach statistical significance in the randomised phase. This suggests its utility, as a marker of successful VIT, is limited.

It is interesting that significant reductions in IL-4 production to non-specific stimulation (PMA/ionomycin) occurred in both VIT and placebo groups. This suggests the possibility of a confounding seasonal effect on results, related to an effect of light exposure on immune system function,[416] or an unidentified change in laboratory conditions or reagents.

11.2.2 Study strengths & weaknesses

The main strengths of this study were its randomised placebo-controlled design and use of a diagnostic sting challenge (incorporating two stings) as the gold standard for determining treatment efficacy and the use of commercially available quality-controlled test kits. The concurrent placebo-controlled design controlled for the influence of confounding variables such as a natural history for reduced sensitivity over time and seasonal changes in TH-1 to TH-2 balance.

Limitations include that:

(i) For logistical reasons tests could not always be performed;

(ii) Laboratory assessments were confined to the initial 3 month period of immunotherapy, therefore changes in the longer term can not be excluded;

(iii) As discussed above (10.2.2) a simple approach was used to assess IL-4 release in unselected cell culture, using a venom concentration for lymphocyte cytokine production based on highest proliferative responses. Methods that select venom-reactive cells prior to non-specific stimulation

may be more useful,[313] and other venom concentrations may improve test performance.

The effect of the significantly higher HRT reactivity in the placebo group is difficult to ascertain. A bias towards having individuals with a higher reaction risk in the placebo group may have reduced the number of highly allergic people with marked laboratory changes during VIT in the randomised phase of the trial, with more of such subjects receiving VIT during the crossover phase.

The high efficacy of VIT would have hindered testing a candidate marker for successful VIT. However, as no *in vitro* test consistently parallelled successful VIT, we were able to conclude that no such marker had emerged.

11.2.3 Comparison with related studies

T cell responses are thought to play a central role in hyposensitisation.[417] T cell proliferation has been found to fall transiently soon after rush VIT, rising again by 1 month then declining again over the following 12 months. No significant change occurs with conventional VIT until one year of therapy has elapsed.[348] With both rush and conventional VIT, T helper (TH) cell cytokine production changes from an IL-4 dominated (TH-2 type) to an IFN-γ dominated (TH-1 type) response to antigen.[313,348,352] This occurs within a few weeks of rush VIT and about 2 months after commencing conventional VIT.[348] Our results were consistent with these previous studies.

A TH-2-type response may be favoured by higher antigen doses,[361,418] and the expression of co-stimulatory molecules by antigen presenting cells.[419] A number of studies have linked IL-10 and IL-12 to the changes in the proliferative and TH-2/TH-1 responses to venom antigens seen during VIT,[176,349,420] and IL-10 in biopsies of cutaneous late-phase reactions in patients undergoing VIT has been noted to increase with therapy.[421] The switch to TH-1 response causes a switch from venom-specific IgE to IgG4 antibody production by B cells.[351] However the levels of these antibodies appear to bear little relationship to clinical protection,[52,237,335] and VIT failure occurs even with very high specific IgG4 levels.[269,322,333] It has been proposed that other factors, such as chemokines produced by T cells, may influence basophil and mast cell reactivity, thus providing a link between the TH-2-TH-1 switch and hyposensitisation.[417] Partial basophil degranulation and mediator depletion in the early phases of rush desensitisation may also play a role.[342-344,354]

Interestingly, the only consistent and significant changes in laboratory parameters during VIT other than IL-4 production were the increase in venom-specific IgE and a

reversal of the trend towards reduced HRT values seen in those not receiving therapy. It may be inferred from this finding that the factors involved in conferring protection in untreated VST positive people (where protection was associated with lesser degrees of histamine release) may be different to the mechanisms by which VIT has an effect. Alternatively, the HRT may simply be a surrogate marker of another process, with the effects of this process on the HRT being over-ridden by the increase in venom-specific IgE that occurs during VIT. The mechanisms that determine sensitivity, and by which VIT confers protection, are probably so diverse that no single marker may be sufficient to determine risk or to monitor the effects of therapy.

11.2.4 Interpretation

None of the in-vitro tests examined here were reliable markers of successful immunotherapy in the three-month period we examined. Lymphocyte proliferative responses and cytokine release patterns designed to assess the TH-1/TH-2 balance have been used to assess the efficacy of novel immunotherapies.[367,422] However, our findings, as well as the uncertainty regarding immunotherapy mechanisms, indicate that no laboratory marker is yet able to replace deliberate challenge with allergen. A possible seasonal effect on results underlines the importance of including a randomised concurrent control group in any study of immunotherapy mechanisms.

11.2.5 Unanswered questions

Whether methodologies that use venom-selected lymphocyte cultures or different venom concentrations will perform better than the tests examined here is unknown. However, until the mechanisms of hyposensitisation are better understood, a useful laboratory marker of treatment efficacy may prove elusive.

Chapter 12: Conclusion

12.1 Major findings

Deaths

All four deaths identified from coronial records occurred in adult males with significant cardiac comorbidities &/or taking medications that may have contributed to death. Three had sought medical care for ant sting allergy previously.

Population prevalence and ED presentations

The population prevalences of JJA, honeybee, *V germanica* and *Myrmecia forficata* sting allergy were 2.7%, 1.4%, and 0.6% and 0.3% respectively. *M pilosula* allergy prevalence increased with age ≥35 (OR 2.4) and bee sting allergy (OR 16.9). In the ED and allergic volunteer groups, those ≥35 had a greater risk of hypotensive reactions (OR 2.9) when last stung. Annual sting exposure rates were 12%, 7% and 2% for JJA, honeybee and *V germanica* respectively. Similarly, ED presentations with JJA anaphylaxis were double that for honeybee, and allergic reactions to *V germanica* and *M forficata* were infrequent.

Allergic volunteers- initial assessment and prospective follow up

RAST had acceptable overall sensitivity (0.9) and specificity (0.8) for the diagnosis of JJA allergy using a low tracer uptake threshold (>0.3%), but non-allergic people with recent (<12 months) sting exposure had a high rate of positive RAST (71%). There appeared to be significant IgE cross-reactivity with other *Myrmecia* venoms, but not with honeybee or wasp venoms. During follow-up, 79 (70%) of 113 jack jumper stings caused systemic reactions. Only prior worst reaction severity predicted the severity of follow-up reactions, with the majority experiencing similar or less severe reactions when stung again.

Efficacy and tolerability of VIT

Sixty-eight healthy volunteers (aged 20–63 years) who were allergic to JJA venom were randomised to placebo (33) and VIT (35). Four on placebo were stopped early and 12 on VIT had their treatment allocations revealed before the sting challenge, thus 29 on placebo and 23 on VIT were included in the primary analysis. Objectively defined systemic reactions to sting challenges arose in 21 of 29 participants (72%) on

placebo and none of 23 on VIT ($p<0.0001$). Of the remaining 12 on VIT who underwent sting challenges after treatment allocations were revealed, only one reacted to sting challenge with transient urticaria that did not require treatment. After crossover of the placebo group to VIT, one of 26 had a reaction to sting challenge (transient urticaria). In all patients who had VIT, objective systemic reactions to therapy were recorded in 22 of 64 (34%) during VIT; two of which were hypotensive.

Sting anaphylaxis

Twenty-two participants had systemic reactions to deliberate sting challenge, of which 19 received interventions according to our protocol. All were given adrenaline, and five received volume resuscitation. In nine cases, physical signs recurred after initial attempts at stopping adrenaline but resolved after recommencing the infusion. The median total dose and infusion duration were 590 ug and 115 minutes respectively, but were significantly higher for eight patients who had hypotensive reactions (762 ug and 169 minutes respectively).

Hypotension was always accompanied by a relative bradycardia, which was severe and treated with atropine in two patients. Widespread T wave inversion occurred, before commencing treatment with adrenaline, in one person with an otherwise mild reaction.

Eleven subjects had reactions that satisfied clinical criteria for severe hypersensitivity (anaphylaxis), corresponding to Mueller sting reaction grades III and IV, for which peak tryptase readings had sensitivity 0.36 and specificity 0.93 using the recommended cut-off range (<12.0 ug/L). Receiver-operator curve analysis suggested a cut-off of 9.0 ug/L would improve diagnostic performance (sensitivity 0.55, specificity 0.93). Serial tryptase measurement was significantly more discriminatory; an increase in tryptase of 2.0 ug/L or greater had a sensitivity of 0.73 and specificity 0.98 The addition of histamine measurements, defining a positive result by either a rise in tryptase or a rise in histamine, appeared to further increase sensitivity (0.90).

In vitro diagnosis of venom allergy and monitoring immunotherapy

Only VST and HRT identified those at risk of sting anaphylaxis in the placebo group. Although IgE RAST, leukocyte SI and IL-4 production, LRT and BAT all correlated well with intradermal venom skin tests, they did not predict sting challenge outcome. After successful VIT, venom-induced leukocyte IL-4 production tended to fall, whereas IgE RAST increased and a natural decline in HRT reactivity was reversed.

12.2 Interpretation

Sting allergy prevalence is determined by age and exposure rate. JJA sting exposure and allergy prevalence in Tasmania are excessive compared to mainland Australia and there is a high risk of systemic reactions in allergic people on re-sting. Prior worst reaction severity and age predict reaction severity and may be used to guide management. Older adults with a history of severe reactions appear to be at greatest risk of severe JJA sting anaphylaxis, and the risk of death is probably further increased by the presence of co morbidities.

Carefully titrated and monitored intravenous adrenaline combined with volume resuscitation is an effective strategy for treating sting anaphylaxis, however severe bradycardia may benefit from additional treatment with atropine. Cardiac effects of anaphylaxis, perhaps including neurocardiogenic mechanisms, may be an important factor in some lethal reactions. Caution is required when using serum tryptase to refute or support a diagnosis of anaphylaxis. Serial tryptase measurement increases sensitivity and specificity.

Using an appropriate diagnostic threshold, detection of venom-specific IgE by RAST appears to be a sensitive test for JJA allergy, but lacks specificity. IgE cross-reactivity between JJA and other *Myrmecia* venoms has significant diagnostic and clinical implications, particularly for mainland Australia where a number of different *Myrmecia* species may coexist in an area. The HRT warrants further assessment for selecting those who stand to benefit most from VIT. Uninformative performance of the commercially available LRT and BAT tests may be due to pre-incubation with IL-3. None of the tests evaluated appear to be reliable markers of successful immunotherapy.

In well motivated, highly allergic but otherwise healthy adults, VIT is highly effective in prevention of JJA sting anaphylaxis. The risk of systemic reactions during VIT requires treatment to be administered with immediate access to resuscitation facilities.

Appendix: Published papers

Brown SGA, Wu QX, Kelsall GR, Heddle RJ, Baldo BA. Fatal anaphylaxis following jack jumper ant sting in southern Tasmania. *Medical Journal of Aust* 2001;**175**(11-12): 644-7.

Brown SGA, Franks RW, Baldo BA, Heddle RJ. Prevalence, severity, and natural history of jack jumper ant venom allergy in Tasmania. *Journal of Allergy and Clinical Immunology* 2003;**111**(1):187-92.

Brown SGA, Wiese MD, Blackman KE, Heddle RJ. Ant venom immunotherapy: a double-blind, placebo-controlled, crossover trial. *Lancet* 2003;**361**(9362):1001-6.

Brown SGA, Heddle RJ. Prevention of anaphylaxis with ant venom immunotherapy. *Current Opinion in Allergy and Clinical Immunology* 2003; **3**(6):511-6.

Brown SGA, Blackman KE, Stenlake V, Heddle RJ. Insect sting anaphylaxis; prospective evaluation of treatment with intravenous adrenaline and volume resuscitation. *Emergency Medicine Journal* 2004;**21**(2):149-154.

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