

ORIGINAL PAPER

Defining the T cell antigen proteome of wasp venom

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Summary

Background While modulation of T cell function is believed to be important in the successful acquisition of clinical tolerance during venom immunotherapy, little is known of the role of wasp venom specific T cell antigens.**Objective** We sought comprehensively to characterize the T cell proteome for wasp venom to facilitate the future development of T cell-based immunotherapeutic approaches.**Methods** Using peripheral blood mononuclear cells from wasp venom-allergic individuals and IL-4 ELISPOT analysis, we characterized T cell responses to whole venom and gel filtration/ion exchange-fractionated venom. Reactive fractions were purified and identified using highly sensitive electrospray ion-trap mass spectrometry.**Results** Wasp venom-allergic individuals have detectable whole wasp venom-specific T cells directly *ex vivo*, which show rapid IL-4 effector function. T cell responses to gel filtration/ion exchange fractionated venom were dominated by responses to phospholipase A₁, hyaluronidase and antigen 5.**Conclusion** Although it is likely that there are many T cell antigens within wasp venom, the main responses are to proteins coincident with the known IgE-binding proteins.**Keywords** elispot, gel-filtration/ion exchange chromatography, proteome, wasp allergy*Submitted 24 May 2006; revised 27 June 2006; accepted 25 July, 2006*

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Introduction

The administration of escalating doses of wasp venom is a highly effective approach to induce clinical tolerance in wasp venom allergic individuals [1]. An understanding of the underlying mechanisms is essential for the development of new strategies for the generation of acquired peripheral tolerance for venom allergy and other clinical hypersensitivities. Successful immunotherapy of human allergic disease is associated with a number of molecular and cellular events. For example, increases in allergen-specific IgG (e.g. IgG4) and late reductions in specific IgE have been described [2–4]. In addition, many studies have shown that there is a relative reduction in tissue mast cells and eosinophils associated with immunotherapy [5–13]. Changes in cytokine production by CD4⁺ and CD8⁺ T cells are now well documented and although there are differences between study systems, these changes may include: reduction in T-helper type 2 (Th2) cytokines; elevation in Th1 cytokines; and elevation in IL-10, regulatory T cells and/or transient anergy [5, 14–46]. Overall these data are

consistent with a central role for changes in T cell function for acquisition of clinical tolerance to allergens.

Whole allergen immunotherapy carries a risk of IgE-mediated anaphylaxis and therefore attention has focused on the development of T cell peptide-based approaches [19, 37, 47–54]. While T cell peptide immunotherapy does carry associated risks including the induction of late asthmatic reactions in allergic individuals, it is anticipated that optimization of the approach will lead to the development of efficacious and low risk preventive and therapeutic strategies. Identification of the dominant allergens recognized by T cells is clearly important for the understanding of disease pathogenesis and the further development of T cell immunotherapy. While previous attention has focused on the identification of T cell responses to existing IgE-binding proteins, it is possible that T cells recognize other non-IgE-binding proteins within a complex source of allergen such as wasp venom. The immunotherapeutic use of peptides derived from such proteins may further minimize the risk of desensitization-induced

IgE-mediated anaphylaxis, while still being able to induce clinical tolerance to whole venom through linked suppression. Alternatively, the ability to exclude the significant presence of T cell reactive but non-IgE reactive proteins would favour the future development of T cell peptide immunotherapeutic approaches based on peptides or proteins derived from the IgE-binding allergens.

We, therefore, sought to undertake a comprehensive proteomic analysis of the dominant T cell antigens within a defined allergen source to investigate whether they are coincident with known dominant IgE-binding proteins. Wasp venom is a complex mixture of approximately 20 proteins and other material. The main IgE-binding components are thought to comprise phospholipase A₁, hyaluronidase and antigen 5 [55, 56]. Few existing studies have addressed potential T cell antigens within wasp venom, but data suggest that antigen 5-specific T cells are present within the peripheral blood of allergic individuals [57]. Using whole wasp venom, we first investigated whether wasp-venom-specific T cells could be identified *ex vivo* within the peripheral blood of allergic individuals. We then proceeded to fractionate the wasp venom by gel filtration and ion exchange and then to identify the T cell reactive components through electrospray ion-trap mass spectrometry.

Methods

Subjects

Twelve individuals with a clinical history of wasp venom systemic allergic reactions and positive wasp venom specific skin tests and/or circulating IgE detected by RAST were recruited through the venom clinic at the Churchill Hospital under ethical approval from the Oxford Clinical Research Ethics Committee; their clinical details and the severity of their reactions to wasp stings using Brown's [58] grading are given in Table 1. In addition, we recruited seven non-allergic healthy controls. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood using Lymphoprep (Nycomed, Roskilde, Denmark) density gradient. PBMCs were then washed in RPMI supplemented with penicillin, streptomycin and L-glutamine (R0) and resuspended in RPMI with penicillin, streptomycin, L-glutamine and 10% fetal calf serum (FCS; R10).

Table 1. Clinical details of wasp-allergic patients

Patient	1	2	3	4	5	6	7	8	9	10	11	12
Age	61	52	44	56	45	57	42	40	34	53	37	53
Sex	M	M	F	F	M	M	M	F	M	F	F	M
Severity: Grade 1–3	2	3	3	2	3	3	2	3	2	3	3	2

Systemic hypersensitivity reactions to wasp stings are graded according to Brown [58].

ELISPOT

A total of 200 000 PBMCs were added in duplicate wells to a volume of 100 µL of RPMI-1640 and 5% FCS (R10) in 96-well U-bottomed plates (Corning Inc., Corning, NY, USA), one hundred microlitres of *Vespula vulgaris* venom (Vespa Labs, Spring Mills, PA, USA), at a concentration of 10, 5 and 0.5 µg/mL, or 100 µL of the wasp venom fractions was added to the PBMC. The plates were incubated overnight at 37 °C and 5% CO₂ and the cells washed twice with (R10). The cells were then transferred to ELISPOT plates (Millipore Corp, Bedford, MA, USA) that had been coated overnight with anti-human IL-4 Ab (Mabtech, Nacka, Sweden), washed six times and subsequently incubated with R10 at 37 °C. The cells were removed after a further overnight incubation at 37 °C and 5% CO₂ and the plates were washed six times with phosphate-buffered solution (PBS) Tween 0.05% and incubated with a second biotinylated Ab to human IL-4 (Mabtech) for 2 h at room temperature. The plates were washed six times with PBS-Tween 0.05% and incubated with streptavidin-alkaline phosphatase (Mabtech) for 1 h. After a final washing the plates were developed with an alkaline phosphatase substrate (Bio-Rad, Hercules, CA, USA) and the spots were counted using an automated ELISPOT reader. Results were expressed as spot-forming cells per total number of PBMCs after subtracting the background (cells alone).

Protein separation

Ten milligram *Vespula vulgaris* venom (Vespa labs) was dissolved in 1.2 mL of 20 mM Tris 150 mM NaCl pH 8.0 and gel filtration was performed using a Superdex 200 26/60 column on an AKTA Purifier (GE Amersham, Uppsala, Sweden). Fractions were used in the ELISPOT assay. Pooled fractions were buffer-exchanged into 50 mM NaPO₄ pH5 and passed over a HiTrap-SP HP cation exchange column. The column was eluted with a linear gradient of 50 mM NaPO₄ 1 M NaCl pH 5.

Protein identification by tandem mass spectrometry

Fractions were separated on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie-stained bands were excised and digested with trypsin as described previously [59]. Peptides were separated and analysed by nanoLC-MS/MS, using a reversed-phase liquid chromatography system (Ultimate™, Dionex/LC-Packings, Sunnyvale, CA, USA) coupled to an ion trap tandem mass spectrometer (HCTplus™; Bruker Daltonics, Bremen, Germany).

For the separation with a C18 pepmap column (75 µm ID, 10 cm; LC-Packings, Dionex, NL, USA), a flow rate of 200 nL/min was used generated by a cap-flow splitter cartridge (1/100) and an initial pump flow rate of

200 μ L/min. Raw chromatography data were processed and Mascot compatible files created using Data AnalysisTM 3.2 software (Bruker Daltonics). Searches were performed using MascotTM software (Matrixscience, London, UK) and the SwissProt (09.2005) and NCBI nr (09.2005) databases [60]. The interpretation and presentation of MS/MS data was performed according to published guidelines [61].

Results

Identification of functional wasp venom-specific T cells *ex vivo*

Previous studies have suggested that antigen 5-specific T cells circulate at relatively low frequency in allergic individuals and are only detectable after proliferation *in vitro*. We first investigated whether the frequency of whole venom-specific T cells was sufficiently high to be detectable *ex vivo* and whether the reactive cells showed rapid effector function. Using *ex vivo* IL-4 ELISPOT to detect whole wasp venom-specific T cells, we showed that allergic individuals have circulating frequencies up to 700 IL-4 producing wasp venom-specific T cells per million PBMC (Fig. 1), which is within a similar order of magnitude to responses that have been observed to other allergens [62]. In contrast non-allergic controls did not have detectable wasp venom-specific IL-4 producing T cells *ex vivo*. CD4⁺T cell depletion confirmed that the responses were largely mediated by CD4⁺T cells (data not shown). Having established that whole wasp venom-specific T cells could be detected without expansion *in vitro*, we were able to proceed to fractionate the venom and characterize the T cell reactive fractions.

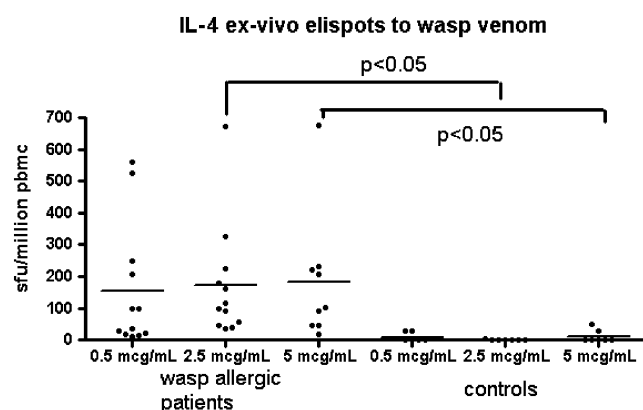


Fig. 1. Peripheral blood mononuclear cells (PBMC) from wasp venom-allergic patients and controls were incubated with *Vespula vulgaris* venom at 5, 2.5 and 0.5 μ g/mL and IL-4 detected as spot forming units (SFU) per million PBMC. The wasp venom allergic patients had significantly higher frequencies of IL-4 producing cells than controls.

Size- and charge-based separation of wasp venom

Whole wasp venom was fractionated by size exclusion gel filtration chromatography (Fig. 2a) and showed several peaks that were dominated by material of less than 47 kDa. Each of the fractions was investigated for T cell reactivity in the IL-4 ELISPOT using freshly isolated PBMC from three wasp-allergic individuals. The fractions that induced the highest frequencies of IL-4 producing T cells corresponded with fractions D5-E10 (Fig. 2b). Three separate pools were constructed from these fractions: pool 1, fractions D5-D10; pool 2, fractions D12-E3; and pool 3, fractions E4-10. Each pool was further separated by ion-exchange chromatography and the resultant fractions were again used in IL-4 ELISPOT assays with PBMC from three wasp-allergic patients (Figs 3 and 4). Fractions A8, A10 and A 11 in pool 1 and fractions A5-A10 in pool 2 produced high frequencies of IL-4 producing T cells when incubated with PBMC from wasp-allergic patients (Figs 3b and 4b). These fractions were therefore further separated by SDS-PAGE and three major bands were observed whose presence correlated with the magnitude of antigen responsiveness of patient derived T cells (Figs 3c and 4c).

Identification of wasp venom antigens by tandem mass spectrometry

We, therefore, proceeded to identify the three main polypeptides observed in these fractions by tandem mass spectrometry. The three bands were excised from coomassie/silver-stained gels, digested with trypsin and analysed by nano-LC-MS/MS as described in Materials and methods. Database searching of the generated MS/MS-specific data identified the bands as phospholipase A₁, hyaluronidase and antigen 5, the known major IgE binding proteins. Thus, in a comprehensive analysis of *ex vivo* T cell responses to wasp venom in allergic individuals, the dominant T cell antigens co-localized to the known IgE-binding proteins.

Discussion

We have shown that wasp venom-allergic individuals have wasp venom-specific T cells which are detectable *ex vivo* and which show rapid IL-4 effector function. The strength of these responses permitted a comprehensive proteomic analysis of the responses to gel filtration/ion exchange-fractionated venom constituents and subsequent characterization of the dominant T cell-reactive fractions. We identified phospholipase A₁, hyaluronidase and antigen 5 as the principle T cell targets within wasp venom. These proteins are identical to the main IgE-binding components of wasp venom suggesting that T cell reactive but non-IgE reactive proteins are rare. Thus, it is unlikely that the use of such purely T cell reactive proteins

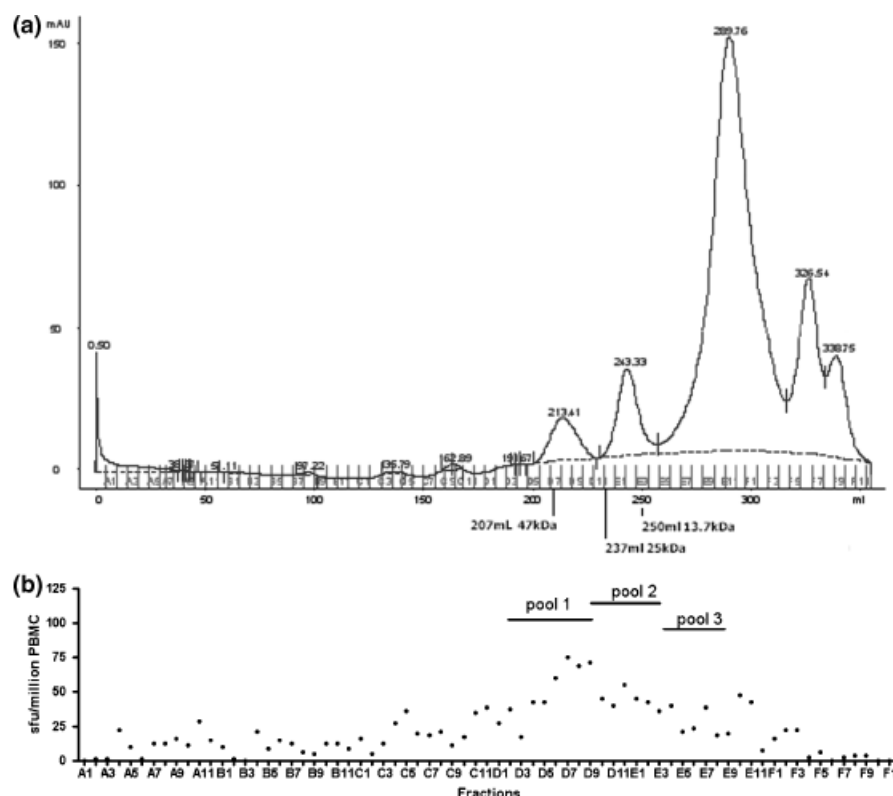


Fig. 2. (a) Gel filtration profile of whole wasp venom generated fractions; (b) peripheral blood mononuclear cells (PBMC) from three wasp-allergic patients were incubated with wasp venom fractionated by gel filtration. The highest frequency of IL-4 secreting cells were detected when PBMC were incubated with fractions D3–E3. Indicated fractions were pooled to create three pools: pool 1, pool 2 and pool 3.

will be feasible for T cell-based immunotherapeutic approaches.

We sought to characterize the T cell proteome from an allergen source in order to facilitate future developments of T cell-based immunotherapy. Although, we had predicted that the dominant T cell antigens would overlap with the known IgE-binding proteins, we wished to seek definitive evidence using a non-biased systematic approach, as the findings would have such significant implications for future development of immunotherapy in general. The presence of purely T cell-reactive proteins would have opened the possibility of using these proteins or derivative peptides to induce linked suppression to the distinct IgE-binding proteins within wasp venom. Linked suppression is thought to account for the loss of responsiveness to different epitopes within a protein or allergen source, following the induction of T cell tolerance using one or more immunodominant epitopes. This form of tolerance may be mediated via an antigen non-specific cognate interaction with antigen presenting cells and/or through the production of immunosuppressive cytokines such as IL-10 and TGF- β possibly from regulatory T cells [42]. In contrast, we have shown that the dominant T cell-reactive components of wasp venom are coincident with the known IgE-binding proteins.

The current study confirms that it is possible to investigate the T cell proteome of an antigen source in an unbiased approach that will allow detection and identification of the main T cell reactive components. By studying fresh *ex vivo* responses, we have excluded the possibility that the relative antigenic T cell immunodominance was altered during expansion *in vitro*. Furthermore, the combined size and charge separation achieved with gel filtration/ion exchange chromatography and PAGE permitted the generation of extremely pure fractions that were amenable to analysis by the highly sensitive electrospray ion-trap mass spectrometry. We hope that the documentation of this approach will also be useful in the many other diseases in which it has not proved possible to use antibody binding to guide the identification of unknown T cell target antigens, for example in some putative T cell-mediated auto-reactive diseases such as psoriasis and B27-associated rheumatological disease.

In summary, we have used T cell antigen proteomics to show that the dominant T cell targets within wasp venom are similar to the known IgE-binding proteins. By excluding the significant presence of T cell reactive but non-IgE reactive proteins, we would anticipate that induction of linked suppression based on such purely T cell reactive fractions is unlikely to be possible. Instead it seems reasonable to continue the development of T cell

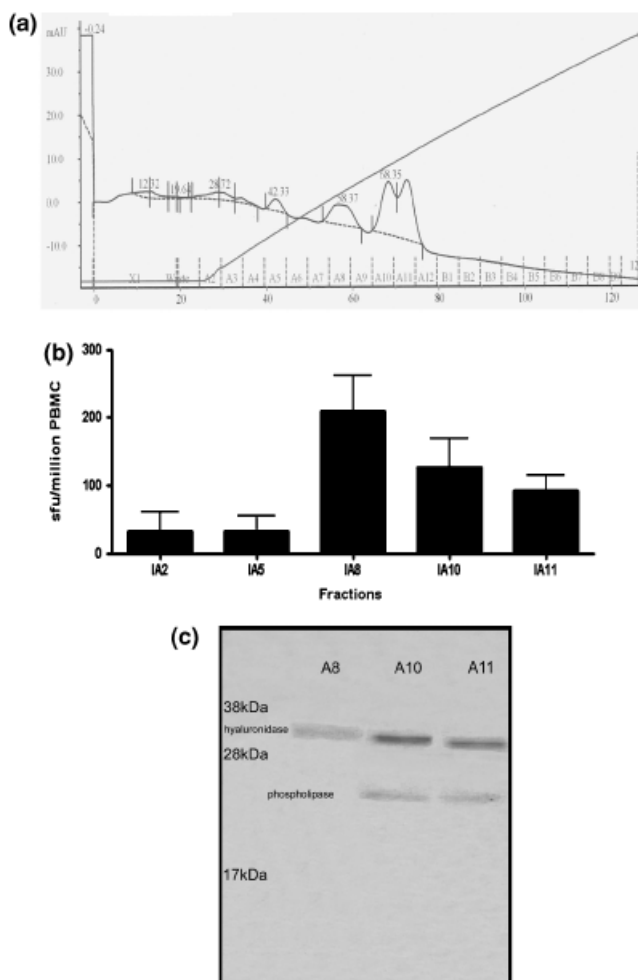


Fig. 3. Pool 1 (a) was further fractionated by ion-exchange chromatography; (b) fractions that corresponded with peaks of absorbance were incubated with peripheral blood mononuclear cells (PBMC) from three wasp allergic patient and IL-4 detected as spot forming units (SFU) per million PBMC; (c) sodium dodecyl sulphate-polyacrylamide gel electrophoresis of fractions A8, A10 and A11 from pool 1.

immunotherapeutic approaches using peptide or protein sequences from the known existing IgE-binding proteins.

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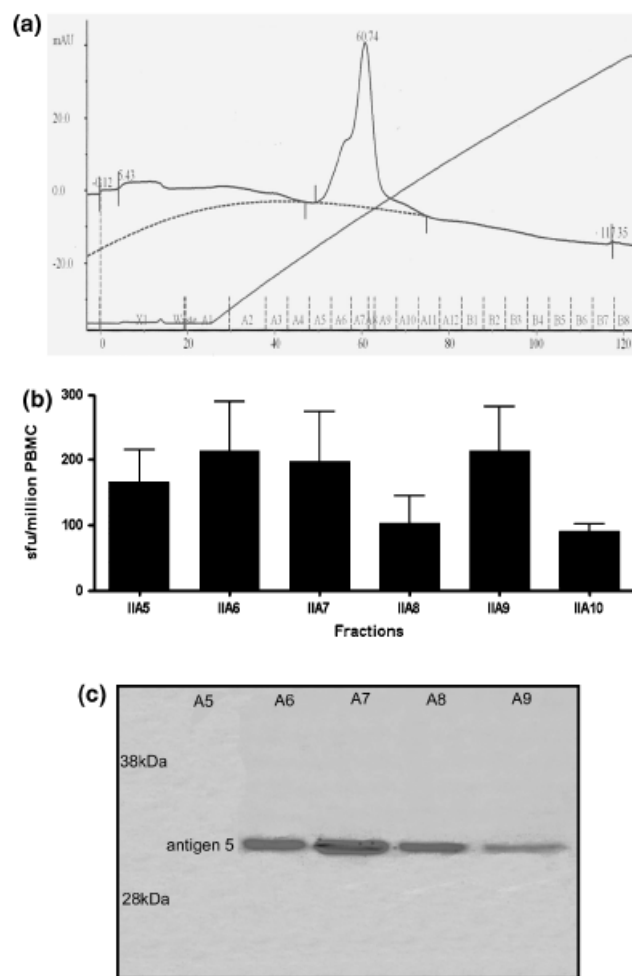


Fig. 4. Pool 2 (a) was further fractionated by ion-exchange chromatography; (b) fractions that corresponded with peaks of absorbance were incubated with peripheral blood mononuclear cells (PBMC) from three wasp allergic patient and IL-4 detected as spot forming units (SFU) per million PBMC; (c) sodium dodecyl sulphate-polyacrylamide gel electrophoresis of fractions A5–9 from pool 2.

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