

Characterization of proliferative responses and cytokine mRNA profiles induced by *Vespula* venom in patients with severe reactions to wasp stings

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SUMMARY

The reasons why severe allergic reactions to bee and wasp stings develop in only a small portion of exposed individuals are incompletely understood, but differences in T cell responses to venom antigens comparing allergic and non-allergic individuals are likely to be important. To identify such differences, venom-induced proliferative responses and cytokine mRNA production by blood mononuclear cells from *Vespula* venom-allergic patients and non-allergic individuals were compared. Mononuclear cells from most venom-allergic patients proliferated in response to alkylated *Vespula* venom (7275 ± 8387 ct/min, $n = 19$), and the extent of proliferation was greater for patients with a history of multiple prior stings and those with high levels of venom-specific IgE. Although mononuclear cells from non-allergic subjects showed little or no proliferation in response to venom (926 ± 711 ct/min, $n = 8$), production of mRNAs coding for IL-2, IL-4, IL-5, IL-10 and interferon-gamma (IFN- γ) in response to *Vespula* venom by cells from non-allergic subjects was detected by reverse transcriptase-polymerase chain reaction (RT-PCR), indicating that these individuals had been previously sensitized to venom antigens. In contrast to the Th0 cytokine mRNA profile observed for non-allergic individuals, venom-allergic patients released a more restricted profile of cytokines following stimulation with venom. Only IFN- γ mRNA expression was detected in all individuals evaluated, whereas IL-2 mRNA was not detected during the first 48 h of stimulation, and T cells from only one of three venom-allergic individuals produced detectable IL-4 or IL-5 mRNA. The difference in cytokine profiles observed comparing venom-allergic patients and non-allergic controls could not be attributed to intrinsic differences in T cells from these individuals, because polyclonal stimulation with phorbol myristate acetate (PMA) + ionophore induced similar cytokine mRNA profiles in the two groups. These studies demonstrate clear differences in the T cell responses of venom-allergic subjects, that may contribute to the development of severe allergic reactions in these individuals.

Keywords allergy wasp venom cytokine mRNA RT-PCR

INTRODUCTION

Allergic reactions to bee or wasp stings occur frequently in humans. Severe generalized reactions occur in up to 0.8–2% of the adult population in Europe and the USA, and because of the risk of laryngeal oedema or anaphylactic shock following re-sting, which can lead to death or major disability, these individuals require venom immunotherapy, frequently for prolonged periods of time [1–3].

The pathogenic mechanisms responsible for bee and wasp sting allergy, and the reasons why such allergies develop in only a

portion of the population, are incompletely understood. The production of high levels of IgE antibodies recognizing components of bee venom is strongly associated with systemic reactions, but some patients without venom-specific IgE antibodies can develop serious reactions to re-sting and, in other patients, these antibodies can persist after successful immunotherapy [4,5]. Thus, additional factors, such as the presence or absence of 'blocking' IgG antibodies recognizing venom components, and the extent that venom induces the release of mediators by inflammatory cells and platelets, participate in determining the type and severity of systemic reactions that occur [5,7]. Considerable evidence indicates that cytokines produced by T lymphocytes in response to specific antigens/allergens are extremely important in controlling both the functional properties of inflammatory cells and isotype of

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antibodies produced by B cells [8,9]. Thus, the types of cytokines produced by T cells responding to venom antigens are likely to be extremely important in the pathogenesis of venom allergy.

Marked differences in the patterns of cytokine production can be observed in different T cell populations. CD4⁺ T cells are commonly divided into Th1 cells, which produce IL-2, interferon- γ and lymphotoxin- α , and Th2 cells, which produce IL-4, IL-5, IL-6 and IL-10 [10]. In addition, Th0 cells, thought to be the progenitors of Th1 and Th2 cells, have been shown to produce all of these cytokines. These different T cell subpopulations, originally described in mice, have also been shown to exist in man. Recently, a number of studies have suggested that T cells producing Th2 cytokines are important in the induction and maintenance of allergic responses [11], and that immunotherapy can modulate the relative proportion of Th1 and Th2 cytokines in several different allergic conditions [12,13].

Less information is available concerning the profile of cytokines produced by T cells in response to bee and wasp venoms, at least in part because of two technical problems that have complicated such analyses. First, attempts to activate T cells with bee and wasp venoms have been limited by the toxicity of these preparations, and techniques that remove these cytotoxic activities while preserving antigenicity have only recently been described [14]. Second, a very low proportion of total T cells are activated by allergens such as bee and wasp venoms, and assays that are sufficiently sensitive to detect cytokine production immediately following activation with venom antigens are not available. Consequently, studies reporting cytokine production induced by *Hymenoptera* venom have generally been performed using T cell clones [14] or cell lines that have been stimulated sequentially with antigen and mitogen [15]. Studies evaluating the production of cytokines by T cells in response to wasp venom have not been reported.

In order to compare the proliferative responses and cytokine production of T cells from venom-allergic and non-allergic individuals, we used a non-toxic preparation of *Vespa* venom that induces strong proliferation of T cells from most venom-allergic individuals. To assess the profile of cytokines induced by *Vespa* venom, a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) technique was used that permitted the detection of cytokine mRNAs within the first 48 h after stimulation with this antigen.

PATIENTS AND METHODS

Study populations

Nineteen patients (age 42 ± 16 years) with a prior history of systemic allergic reactions following wasp stings were selected for the study (Table 1). Allergy was documented by a positive skin test for *Vespa* venom (DHS; Bayer Pharma, Puteaux, France) and by the presence of specific IgE (CAP test; Pharmacia, Uppsala, Sweden). The clinical symptoms experienced by these patients after their last wasp sting were classified according to the method of Mueller [16]. Eight healthy volunteers (age 28 ± 3 years) with no personal or family allergic history were evaluated as controls.

Preparation of cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, and had a viability of $> 95\%$ in

Table 1. Clinical profile of *Vespa* venom-allergic patients

Patient	Number of stings	Last sting (months)	Reaction type*	Skin test†	CAP test‡
1	2	12	I	≥ 0.001	3
2	4	12	III	≥ 0.001	2
3	2	10	IV	≥ 0.0001	3
4	2	60	III	≥ 0.1	2
5	2	24	II	≥ 0.01	1
6	1	10	0	≥ 0.01	1
7	3	11	III	≥ 0.01	2
8	1	10	0	≥ 0.001	4
9	6	11	IV	≥ 0.01	3
10	1	120	0	≥ 0.001	3
11	2	24	III	≥ 0.0001	5
12	2	60	IV	≥ 0.01	0
13	2	8	I	≥ 0.001	3
14	3	8	I	≥ 0.001	4
15	2	8	IV	≥ 0.1	2
16	4	36	IV	≥ 0.001	1
17	4	11	II	≥ 0.1	2
18	6	11	III	≥ 0.0001	5
19	3	24	II	≥ 0.001	4

*Reactions are classified as described by Mueller [16].

†Minimum dose of *Vespa* venom ($\mu\text{g/ml}$) able to induce a positive intracutaneous reaction.

‡CAP Test (Pharmacia, Uppsala, Sweden) for serum *Vespa*-specific IgE were graded as follows: 0 = < 0.35 kU/l; 1 = 0.35 – 0.7 kU/l; 2 = 0.7 – 3.5 kU/l; 3 = 3.5 – 17.5 kU/l; 4 = 17.5 – 50 kU/l; 5 = 50 – 100 kU/l; 6 = > 100 kU/l.

all cases. T lymphocytes were purified from the PBMC by depletion of monocytes and B cells by adherence to nylon wool columns, as previously described [17].

Preparation of *Vespa* venom

Because *Vespa* venom proved to be toxic for cells (see below), the venom was denatured according to the method of King *et al.* [18], which was originally described to prepare denatured phospholipase A₂ (PLA₂) from honeybee venom. Briefly, 5.5 mg of lyophilized venom were dissolved in 0.1 M Tris–HCl buffer pH 8.6 and denatured in 6 M guanidine–HCl in the presence of 5 mg/ml dithiothreitol (Sigma Chemical Co., St Louis, MO). After 18 h at 25°C, the reduced product was alkylated by the addition of iodoacetic acid (final concentration 12 mg/ml). After a 20 min incubation, during which the pH was maintained at 8.0 by the addition of 2 M Tris base, the product was dialysed against a 0.1 M ammonium bicarbonate solution for 48 h at 4°C. The protein concentration of the denatured wasp venom was measured according to the method of Lowry *et al.* [19], and aliquots were maintained at -80°C until use.

T cell activation and proliferation assays

To evaluate allergen-induced T cell proliferation, 1×10^5 PBMC were cultured in triplicate in 96-well round-bottomed plates (Falcon 3077; Becton Dickinson Labware, Lincoln Park, NJ) containing 200 μl complete medium (RPMI 1640 containing 2 mM L-glutamine, 200 U/ml penicillin G, 200 $\mu\text{g/ml}$ streptomycin (GIBCO, Cergy, France)) supplemented with 10% human AB serum (Institut Jacques Boy, Reims, France) in the presence of varying

amounts of undenatured or denatured *Vespula* venom. Cultures were incubated for 7 days at 37°C in 95% air/5% CO₂, and proliferation was assessed by measuring ³H-thymidine (1 µCi/well, sp. act. 25 Ci/mmol; Amersham, Les Ulis, France) incorporation during the last 18 h of culture. Preliminary experiments demonstrated that maximal proliferation occurred at 7 days and was obtained using 5 µg/ml of denatured *Vespula* antigen. To evaluate the possible inhibitory activity of the antigen preparations, T cells were activated with 50 ng/ml phorbol myristate acetate (PMA; Sigma) and 1 µg/ml calcium ionophore (A23187; Calbiochem, La Jolla, CA), and the effect of varying doses of venom on the resulting T cell proliferation was determined.

To evaluate the antigen- and mitogen-induced transcription of cytokine mRNAs and cytokine secretion, 5 × 10⁶ PBMC (cytokine production by PBMC) or 1 × 10⁶ PBMC or T lymphocytes (all other assays) were suspended in 1 ml complete medium containing 10% fetal calf serum (FCS; GIBCO), and either 5 µg/ml denatured venom or 50 ng/ml PMA + 1 µg/ml A23187, and cultured in round-bottomed tubes (no. 2005; Falcon) at 37°C (95% air/5% CO₂). After varying times of incubation, cultures were centrifuged (800 g; 10 min), the supernatant was removed, and the cell pellets were washed with PBS. Both supernatants and cell pellets were stored at -80°C until use.

Detection of IL-4 by ELISA

IL-4 in culture supernatants was measured using solid-phase ELISA kit (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Optical density (OD) was measured using a 96-well ELISA reader (ThermoMax; Molecular Devices, Menlo Park, CA); standard curves were linearized by logarithmic transformation. All samples and standards were measured in duplicate.

RNA isolation and cDNA synthesis

Total cellular RNA was extracted from cell pellets using a modification of the procedure of Chomczynski & Sacchi [20]. Briefly, 1 × 10⁶ frozen cells were lysed in the presence of RNazol (Bioprobe Systems, Montreuil-sous-Bois, France) and chloroform, and RNA was precipitated by adding isopropanol and cooling the samples to -20°C for 15 min. The RNA pellet was washed once in 70% ethanol, dried and dissolved in 12.5 µl DEPC-treated water. The total cellular RNA (12.5 µl) was then reverse-transcribed into cDNA by incubation in 30 µl (final volume) of 50 mM Tris-HCl containing 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 450 U Moloney

murine leukaemia virus RT (GIBCO BRL, Life Technologies, Gaithersburg, MD), 15 nmol each of dNTP (Pharmacia Biotech), 60 U RNasin (Promega, Madison, WI) and 1.5 nmol random hexamer primers (pd (N)6; Pharmacia). The reaction was incubated at 42°C for 60 min, heated to 95°C for 5 min to inactivate the RT, and cDNA was stored at -80°C until use.

Quantification of cDNA

To permit the comparison of cytokine mRNAs detected in different samples by RT-PCR, it was important that equivalent amounts of cDNA from each sample were used. Therefore, the amount of cDNA corresponding to either the abundant ribosomal protein S14 (for experiments comparing cytokine production by PBMC) or the δ-chain of CD3 (experiments comparing cytokine production by T lymphocytes) was quantified in each sample, and subsequent comparisons of cytokine cDNAs were made using aliquots of the different samples that contained equivalent amounts of S14 or CD3δ cDNA. S14 and CD3δ cDNAs were quantified by competitive PCR [21,22].

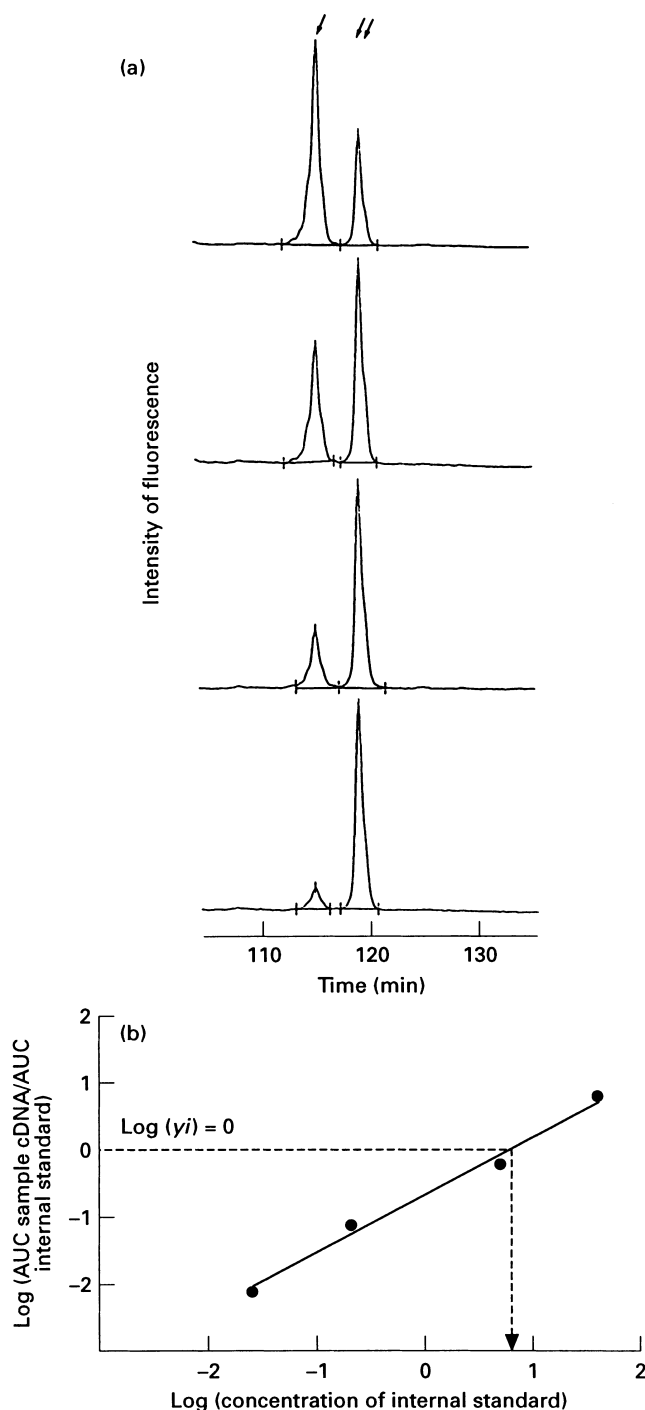
The internal standards required for competitive PCR were obtained by three successive PCR reactions. To produce the S14 standard, a 134-bp fragment of S14 cDNA was amplified using the S14A/S14B primer pair (Table 2). The resulting product was diluted 10 000-fold and reamplified using S14A and the mutational primer S14C (5' GTG CTG TCA GAG GGG ATG GGG CCT CAA TCC GCC CGA TCT TCA), which introduces a 4-bp deletion into the originally amplified sequence [21]. The 130-bp PCR product was purified by electrophoresis into 2% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) and recovered from the gel using a Mermaid Kit (Bio 101, La Jolla, CA). The purified sequence was then reamplified using the S14A/S14B primer pair, and the 130-bp internal standard for S14 was quantified by spectrophotometry. The CD3δ internal standard was produced by an analogous approach using the CD3δA/CD3δB primer pair (Table 2) to amplify a 94-bp fragment of CD3δ, and the mutational primer CD3δC (5' CAT GAG ACT GGA AGG CTG TCT GGG GAC ACA CAA GCT CTG TTG AGG) to introduce a 6-bp deletion, generating a standard of 88 bp.

To measure S14 or CD3δ cDNA content, reaction mixtures containing 10 pmol each of appropriate primers (S14A/S14B or CD3δA/CD3δB primer pairs, respectively), 200 µM of each dNTP, 2 mM MgCl₂, 0.25 U Taq polymerase (Appligene, Pleasanton, CA), 0.1 µl cDNA from each sample and four different amounts of the internal standard (5 × 10⁵, 1 × 10⁵, 5 × 10⁴ and 1 × 10⁴ total copies)

Table 2. Primers and probes used for evaluation of cytokine mRNA profiles by reverse transcriptase-polymerase chain reaction

IL-2	Primers	5' ATG TAC AGG ATG CAA CTC CTG TCT T	5' GTC AGT GTT GAG ATG ATG CTT TGA C
	Probe	5' ACA TGC CCA AGA AGG CCA CA	
IL-4	Primers	5' ATG CGT CTC ACC TCC CAA CTG CT	5' CGA ACA CTT TGA ATA TTT CTC TCT CAT
	Probe	5' TCC AAG AAC ACA ACT GAG AA	
IL-5	Primers	5' TGG CCG TCA ATG TAT TTC TTT ATT AAG	5' TTC TGC ATT TGA GTT TGC TAG CTC T
	Probe	5' ATA GCC AAT GAG ACT CTG AG	
IL-10	Primers	5' GA TGC CCC AAG CTG AGA ACC	5' TAG AGT CGC CACCCT GAT GTC
	Probe	5' CTT TAA TAA GCT CCA AGA GAA A	
IFN-γ	Primers	5' ATG AAA TAT ACA AGT TAT ATC TTG GCT TT	5' GAT GCT CTT CGA CCT CGA AAC AGC AT
	Probe	5' GAC AGA AAA ATA ATG CAG AGC	
CD3δ	Primers	5' CAT GAG ACT GGA AGG CTG TCT G	5' FITC-GAG CAT CAT CTC GAT CTC GGA G
S14	Primers	5' ATC AAA CTC CGC GCC ACA GGA	5' FITC-GTG CTG TCA GAG GGG ATC GGG

were prepared (final volume 50 μ l), and amplified for 30 cycles (S14) or 35 cycles (CD3 δ) using a DNA Thermocycler (Perkin-Elmer, Norwalk, CT). Cycling parameters were as follows: denaturation, 95°C $\times$ 1 min; annealing, 60°C (S14) or 55°C (CD3 δ) for 1 min; and extension, 72°C $\times$ 1 min. The relative amounts of amplification products corresponding to the sample and the internal standard were quantified as previously described [22]. Briefly, appropriate dilutions of PCR products were loaded onto an Automated Laser Fluorescent DNA Sequencer (ALF; Pharmacia) and subjected to electrophoresis. The area under the curve (AUC) of the fluorescent signal corresponding to the sample and internal standard was determined using the ALF manager software (Fig. 1a)



for the four different samples, and this information used to draw the linear regression curve relating the logarithm of the y_i ratio ((AUC sample cDNA)/(AUC internal standard)) to the logarithm of the concentration of the internal standard (Fig. 1b). The concentration of S14 (or CD3 δ) cDNA present in the sample was then obtained from this curve by determining the concentration of internal standard required to give a y_i ratio equal to 1.

To evaluate the reproducibility of this method, CD3 δ cDNA and S14 cDNA in a single sample were quantified repeatedly ($n = 18$) during an 18-month interval of time. The coefficient of variation (s.d./mean $\times$ 100) observed in these studies was $< 35\%$. To test the accuracy of the method, aliquots containing equivalent amounts of cDNA as determined by this approach were used to amplify cDNA coding for the S14 ribosomal (or CD3 δ) protein using the S14A/S14B (or CD3 δ A/CD3 δ B) primer pair. Amplification products were sampled throughout the amplification reaction as described below, electrophoresed into agarose gels and visualized by ethidium bromide staining. As expected for samples containing similar amounts of cDNA, bands of equal intensity were obtained for each specimen, independent of whether the products were obtained during the exponential or plateau phases of the amplification reaction (data not shown). The average amount of CD3 δ and S14 cDNA recovered from 10^6 total cells was not different comparing cells from controls and allergic subjects or as a function of time after activation with antigen or mitogen. In addition, cDNA was prepared from cells after various times of activation with PMA + ionophore, and aliquots containing equal amounts of CD3 δ cDNA were used to evaluate β -actin mRNA expression as described below for cytokines. Consistent with the interpretation that CD3 δ mRNA content remained relatively stable following activation, β -actin mRNA content was similar in samples from unstimulated cells and those activated from 4 h to 72 h prior to evaluation (data not shown).

Quantification of cytokine cDNAs

For amplification of cytokine cDNAs, reaction mixtures (final volume 100 μ l) contained: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5–1.75 mM MgCl₂, 200 μ M of each dNTP, 10 pmol of each oligonucleotide primer (Table 2), 2.5 U Taq Polymerase (GIBCO BRL) and cDNA. For each cytokine, all samples to be compared were amplified during the same PCR, and an equivalent amount of cDNA, quantified as described above, was used in each reaction. Samples were heated to 80°C before adding Taq polymerase to

Fig. 1. Quantification of CD3 δ cDNA by competitive polymerase chain reaction (PCR). Total RNA was purified from T lymphocytes and retro-transcribed into cDNA. Four different amounts of the CD3 δ internal standard were added to aliquots of the cDNA, and a fragment of cDNA coding for CD3 δ was amplified using oligonucleotides CD3 δ A/CD3 δ B. The amplification products were then separated by electrophoresis using an automated laser fluorescent DNA sequencer (ALF; Pharmacia). (a) Elution profiles for aliquots of a single cDNA sample amplified in the presence of (from top to bottom) 5×10^5 , 1×10^5 , 5×10^4 and 1×10^4 total copies of internal standard. Amplification products corresponding to native CD3 δ cDNA (✓) and the internal standard (✓) are indicated. The area under the curve (AUC) for the two peaks was determined using the ALF Manager software. (b) The logarithm of the y_i ratio ((AUC sample cDNA)/(AUC internal standard)) is plotted against the log (concentration of standard cDNA). The concentration of cDNA present in the original sample was obtained by determining the equivalent concentration of internal standard, giving a y_i ratio = 1.

reduce the formation of non-specific amplification products. Cycling parameters were as follows: denaturation, 95°C for 1 min; annealing, 60°C (for IL-4, IL-5) or 55°C (all other cytokines) for 1 min; extension, 72°C for 1 min. On four occasions during the amplification reaction, the thermocycler was maintained at 72°C for the time necessary to remove a 20- μ l aliquot from each reaction mixture, before resuming the amplification reaction. For each cytokine, preliminary experiments were performed in order to define the optimal numbers of cycles for this sampling so that aliquots would be obtained during the exponential phase of the PCR reaction. In most cases, sampling was performed at 25, 30, 35 and 40 cycles. In all experiments, negative controls (buffer without cDNA or samples of mRNA that had been processed as for the RT reaction, but incubated in the absence of enzyme) were included, and always gave negative results. Amplification products were visualized by electrophoresis into 2% agarose gels and visualized by ethidium bromide staining.

To quantify the expression of a given cytokine in the different samples, 10- μ l aliquots of the amplification products obtained at two successive times during the exponential phase of the PCR reaction were added to 190 μ l of 0.4 M NaOH containing 25 mM EDTA and heated for 2 min at 95°C. Samples were rapidly cooled on ice and loaded into the wells of a Minifold Slot blotter (Schleicher & Schuell, Keene, NH) and transferred to Hybond-N nylon membrane (Amersham Corp., Arlington Heights, IL) that had been prewetted in 20 $\times$ SSPE by vacuum aspiration, and each well was washed with 400 μ l of 20 $\times$ SSPE. Membranes were then dried at 80°C for 1 h. For each cytokine, an oligonucleotide probe complementary to a sequence within the amplified fragment (Table 2) was prepared by labelling the 5' end with γ -³²P-ATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (GIBCO BRL). Dot blots were hybridized with the corresponding probe overnight at 50°C (1.5 $\times$ 10⁴ ct/min per ml) and washed as previously described [23]. The radioactivity bound was determined by scintillation spectroscopy (RackBeta LKB; Wallac, Turku, Finland), and results are expressed as ct/min. Quantification by this approach required that under the conditions used, the probe bound only to the specific amplification product. To demonstrate that this was the case, aliquots of the amplification products were electrophoresed into 2% agarose gels, transferred to Hybond-N nylon membranes and hybridized with the labelled probe, as described for dot blots. In all cases, a single band of the appropriate molecular weight was observed (data not shown).

Statistical analysis

Unless otherwise stated, all results are presented as mean $\pm$ s.d. For measurement of cytokine cDNAs, all steps in the procedure (amplification, preparation of dot blots, hybridization and autoradiography) were performed in parallel for all samples to be compared in a single experiment. Proliferative responses and specific radioactivity of the dots from allergic patients and healthy donors were compared using the Mann-Whitney test. $P < 0.05$ was considered significant.

RESULTS

Venom-induced T cell proliferation

No proliferative response was observed when PBMC from allergic patients or controls were stimulated with *Vespula* venom over a wide range of concentrations (0.001–30 μ g/ml). The absence of proliferation appeared to reflect the intrinsic toxic activity of the

preparation, not the presence of suppressor T cells, because the addition of *Vespula* venom strongly inhibited the proliferation of PBMC from both controls and allergic patients induced by PMA + ionophore (data not shown). Following reduction and alkylation, addition of *Vespula* venom in concentrations up to 30 μ g/ml no longer inhibited the proliferative responses of PBMC to PMA + ionophore, indicating that the toxic activity had been entirely eliminated.

PBMC from most, but not all, allergic subjects proliferated in the presence of alkylated *Vespula* venom (Fig. 2). Optimal responses were obtained using venom at a concentration of 5 μ g/ml, and, as was observed for other antigen-induced responses, maximal proliferation occurred at 7 days. Under these conditions, the average proliferative response was 7275 $\pm$ 8387 ct/min ($n = 19$), and 14/19 patients had a stimulation index (SI) of > 2 . In contrast, PBMC from control subjects showed little or no proliferation in response to alkylated *Vespula* venom (926 $\pm$ 711 ct/min, $n = 8$; $P < 0.05$ compared with allergic subjects), and only 2/8 patients had a SI > 2 . Proliferative responses in the absence of added antigen were similar in the two groups (allergic, 1489 $\pm$ 883 ct/min; controls, 804 $\pm$ 216 ct/min; $P > 0.2$).

The five allergic patients presenting with very high levels of serum-specific IgE ($> 17\,500$ U/l) had significantly greater proliferative responses to alkylated *Vespula* venom than those observed for the other patients (14 159 $\pm$ 11 376 ct/min and 4816 $\pm$ 5731 ct/min, respectively, $P = 0.03$). Similarly, the subjects that had been stung three or more times had proliferative responses that were significantly higher than those of patients who were stung on one or two occasions (12 431 $\pm$ 10 527 ct/min ($n = 8$) and 3525 $\pm$ 3511 ct/min ($n = 11$), respectively; $P = 0.02$). In contrast, no associations were observed between the proliferative response to *Vespula* venom and the interval of time since the last sting, the severity of the last reaction judged according to the method of Mueller [16], and results of skin testing.

Cytokine mRNA profiles following mitogen-induced activation

To determine whether lymphocytes from allergic subjects and controls differed in their intrinsic capacity to produce lymphokines, T cells were purified from peripheral blood, stimulated with PMA + ionophore, and the expression of cytokine mRNAs assessed by RT-PCR. To permit comparisons between different individuals, cDNA coding for the ribosomal protein S14 was quantified by competitive PCR in each sample, and aliquots of cDNA from each subject containing equivalent amounts of S14 cDNA were used to evaluate cytokine mRNA content. For all cytokines studied, mRNA expression was extremely low or undetectable before stimulation. In all cases, peak mRNA expression occurred between 6 h (IL-4, IL-5, IL-10) and 12 h (IL-2, IFN- γ) after stimulation.

Following stimulation with PMA + ionophore, expression of IL-4 mRNA could be detected in 3/5 venom-allergic patients and 2/4 control subjects. For every other cytokine tested, stimulation with PMA + ionophore led to detectable expression in every subject in both groups. Overall, no differences were observed comparing the levels of mRNAs for these cytokines in venom-allergic subjects and controls (Table 3).

Cytokine mRNA profiles following allergen-induced activation

In order to evaluate cytokine mRNA expression induced by *Vespula* venom, PBMC were incubated in the presence of denatured *Vespula* venom, RNA was extracted, and cytokine mRNA content was determined by RT-PCR. Kinetic studies demonstrated

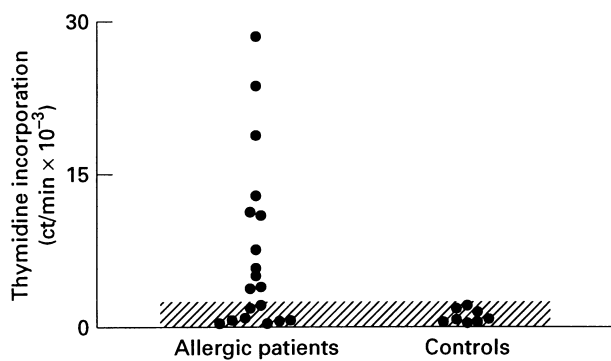


Fig. 2. Peripheral blood mononuclear cells (PBMC) (1×10^5) from venom-allergic patients or controls were cultured in the presence of $5 \mu\text{g/ml}$ denatured *Vesputa* venom for 7 days and thymidine incorporation during the last 18 h of culture was measured. Each point represents the mean of triplicate determinations. ▨, Mean $\pm$ 2 s.d. for proliferation by cells from controls.

that peak cytokine mRNA production induced by venom occurred somewhat later than that induced by PMA + ionophore, and was dependent on the cytokine studied (IL-4, 12 h; IL-2, 24 h; IL-10, 24–48 h; IL-5, 24–48 h; IFN- γ , 24–48 h). The kinetics of cytokine production by PBMC from venom-allergic and control subjects were similar (data not shown). In the absence of stimulation, low levels of IL-10 and IFN- γ mRNA could be detected in cells from some individuals, but no significant differences were observed comparing cells from venom-allergic patients and controls (IL-10, 120 ± 25 and 291 ± 232 ct/min, respectively; IFN- γ , 1939 ± 2301 and 5606 ± 8254 ct/min; $P > 0.2$ for each comparison). No other cytokine mRNAs were detected in unstimulated cells.

The pattern of cytokine mRNA expression by PBMC from venom-allergic subjects stimulated with $5 \mu\text{g/ml}$ denatured *Vesputa* venom was not typical of a Th2-like response. Thus, mRNA for IL-4 and IL-5 could be detected in only one of the three patients studied (Table 4). IL-10 mRNA, although detected in all venom-allergic patients, was expressed to a greater extent by PBMC from non-allergic individuals. mRNA coding for IFN- γ , a prototypic Th1 cytokine, was also produced by cells from allergic patients. It is noteworthy that although PBMC from venom-allergic subjects demonstrated a strong proliferative response to denatured *Vesputa* venom in all cases (thymidine incorporation at 7 days, 4887, 23 623 and 5709 ct/min, respectively, for the patients shown in Table 4), no IL-2 mRNA could be detected by RT-PCR during the first 48 h of culture.

The pattern of cytokine mRNA expression by PBMC from non-allergic controls following stimulation with denatured *Vesputa* venom was strikingly different. Despite the fact that cells from control subjects showed little or no proliferative response to *Vesputa* venom (316 ± 448 ct/min; $n = 3$), mRNA coding for each of the cytokines studied could be detected in every control subject following stimulation with *Vesputa* venom (Table 4). Thus, the cytokine mRNA profile observed in control subjects was typical of a Th0 pattern.

IL-4 could not be detected by ELISA in supernatants of *Vesputa*-stimulated PBMC, independent of whether the cells were obtained from venom-allergic or control subjects.

DISCUSSION

Although a prior history of bee and wasp stings is essentially

Table 3. Expression of cytokine mRNAs by T lymphocytes from venom-allergic and control subjects following stimulation with phorbol myristate acetate (PMA) + ionophore*

Cytokine	Time (h)†	mRNA expression (ct/min $\pm$ s.d.)	
		Venom-allergic	Control
IL-2	12	895 ± 109	682 ± 63
IL-4	6	1178 ± 787	916 ± 1128
IL-5	6	$13\,995 \pm 2603$	7891 ± 6084
IL-10	12	1549 ± 581	1846 ± 1707
IFN- γ	12	5926 ± 4878	2414 ± 821

*Cytokine mRNA expression by T cells from venom-allergic patients ($n = 5$) and controls ($n = 4$) was quantified by RT-PCR as described in Patients and Methods. $P > 0.2$ comparing venom-allergic and control subjects for each cytokine.

†Cells were harvested at the indicated time following stimulation.

universal in adults, only a small fraction of the total population develop severe allergic symptoms. These adverse reactions are thought to result from an uncontrolled immune response directed against venom antigens, but the mechanisms responsible are not well defined. In this study, several differences in the response to *Vesputa* venom by T cells from allergic and non-allergic individuals were identified that may contribute to the development of such uncontrolled responses.

First, T cell proliferation in response to denatured *Vesputa* venom was significantly higher comparing venom-allergic patients and control subjects. Like control subjects, however, T cells from some of the venom-allergic patients did not demonstrate significant proliferation in response to *Vesputa* venom, and this criterion alone could not be used to distinguish allergic and non-allergic individuals. Little previous data on T cell proliferation to bee and wasp venom exist. In most prior studies, undenatured preparations of venom were used as antigen [6,15,24–28]. These preparations contain activities that inhibit lymphocyte proliferation, and, as confirmed in this study, proliferative responses to undenatured venom were generally low or absent. Recently, T cell proliferation in response to PLA₂ of bee venom was evaluated using non-toxic antigen preparations [27]. As observed in our study, proliferative responses were, on average, higher in venom-allergic individuals than non-allergic controls, but only 65% of allergic patients demonstrated significant proliferation to PLA₂.

Our and previous studies provide some insight into the variability in antigen-induced T cell proliferation observed comparing both allergic and non-allergic individuals and within the population of allergic subjects. Clearly, the absence of a proliferative response in some subjects did not result from an absence of prior sensitization to the antigen used to induce proliferation. The *Vesputa* venom used in these studies was a crude extract likely to contain most of the antigenic proteins present in venom, and all but one of the venom-allergic individuals expressed antibodies recognizing these proteins. Similarly, T cells from control subjects showed little or no proliferative response to *Vesputa* venom. These control individuals, however, had been previously sensitized, as demonstrated by the ability of *Vesputa* venom to induce rapid expression of cytokine mRNAs.

We found that venom-induced proliferative responses were higher in patients who had received multiple stings, suggesting that

Table 4. Cytokine mRNA expression induced by stimulation of peripheral blood mononuclear cells (PBMC) with denatured *Vespula* venom*

Cytokine	Time after stimulation (h)	Venom-allergic			Controls		
		Amount of cytokine produced (ct/min)					
IL-2	24	13†	17†	21†	3508	10 555	6912
IL-4	12	34 010	44†	39†	14 555	28 028	14 657
IL-5	24–48	93†	2052	41†	520	8415	2137
IL-10	24–48	1576	540	927	6640	1437	2920
IFN-γ	24–48	38 302	23 833	32 819	36 495	102 113	64 048

*PBMC from venom-allergic and control subjects were incubated in the presence of 5 μ g/ml denatured *Vespula* venom for 12, 24 and 48 h, and cytokine mRNA levels were measured by quantitative polymerase chain reaction (PCR), as described in Patients and Methods. Results are expressed as ct/min for the peak cytokine mRNA expression for each individual. Time after stimulation indicates the time at which peak cytokine mRNA expression was observed.

†No amplification product corresponding to the indicated cytokine was visible on ethidium bromide-stained gels.

extent of prior exposure could influence the extent of proliferation. Nevertheless, some of our patients with a history of multiple stings demonstrated little or no response to *Vespula* venom. In this regard, prior studies have found that beekeepers, despite their extensive exposure to bee venom, have lower venom-induced proliferative responses than allergic subjects, and administration of large amounts of allergen to allergic individuals in the course of desensitization can lead to a decrease in allergen-induced proliferative responses [26,27]. Thus, although the extent of exposure to *Vespula* venom may influence the proliferative response, other factors are clearly important.

Only a very small proportion (<1%) of total peripheral blood T cells specifically recognize allergens such as *Vespula* venom [29]. Consequently, as discussed elsewhere [30], most of the lymphocyte proliferation observed in 7 day *in vitro* proliferation assays results from the activation of bystander T cells through antigen-independent, MHC-unrestricted pathways. Although the mechanisms involved are not clearly defined, the production of soluble lymphostimulatory activities by antigen-activated cells is likely to be involved [30–32]. Thus, not only the number of antigen-specific T cells present, but also the functional capacities of these cells following activation are important in determining the magnitude of the proliferative response that ensues. In turn, the functional capacity of T cells following activation is dependent on numerous factors, including the dose of antigen used during stimulation, the type of costimulatory signals received from accessory cells, and the differentiated state of the T cells before activation. Thus, multiple examples have been described where the extent of antigen-induced lymphocyte proliferation has been shown to be completely independent of other immune activities, such as production of IL-2 and other cytokines or the ability to provide help for immunoglobulin synthesis by B cells [33–35]. Interestingly, we found that the individuals whose T cells proliferated well in response to *Vespula* venom also tended to have higher levels of venom-specific IgE, suggesting that the activities associated with the induction of T cell proliferation may also promote IgE synthesis. Consistent with this idea, a correlation between allergen-induced T cell proliferation and specific IgE levels has also been observed for patients with allergy to house dust mite [36], *Ambrosia* [37] and *Hymenoptera* venom [38]. Thus, our findings suggest that the increased venom-induced T cell proliferation and specific IgE levels observed in venom-allergic subjects reflect differences in the differentiated state of T cells from these individuals compared with that of cells from non-allergic controls. Given the

variability in both lymphocyte proliferation and specific IgE levels within the venom-allergic population studied here, it is probable that there also exists considerable heterogeneity in the functional capacities of the T cells of these individuals.

The evaluation of allergen-induced cytokine mRNA profiles also demonstrated important differences in T cell function comparing venom-allergic and control subjects. Despite the low levels of venom-induced proliferation observed, all control subjects evaluated produced easily detectable amounts of mRNA for IL-2, IL-4, IL-5, IL-10 and IFN- γ . Thus, these individuals all demonstrated evidence of prior sensitization to wasp venom, and the cytokine mRNA profile induced by venom was typical of a Th0-like response. In contrast, T cells from allergic subjects, all of which proliferated in response to *Vespula* venom, produced a much more limited set of cytokines. In particular, IL-2 mRNA was not detected during the first 48 h of stimulation, and T cells from only one of three venom-allergic individuals produced detectable IL-4 or IL-5 mRNA. Only IFN- γ mRNA expression was detected in all individuals. Thus, a Th2 cytokine mRNA profile was never observed when T cells from allergic individuals were stimulated with *Vespula* venom. The difference in cytokine profiles observed comparing venom-allergic patients and non-allergic controls could not be attributed to intrinsic differences in T cells from these individuals, because polyclonal stimulation with PMA + ionophore induced similar cytokine mRNA profiles in the two groups.

In contrast to the findings of this study, several authors have demonstrated that stimulation of peripheral blood T cells from allergic individuals with the pertinent antigen induces a Th2-like response [15,39]. Certainly, the high levels of venom-specific IgE seen in our patients suggests that Th2 cytokines are produced by these individuals *in vivo*, at least in certain compartments. An alkylated antigen was used for these *in vitro* studies, raising the possibility that removing the toxic activity of wasp venom may alter its ability to serve as an allergen. The study of King *et al.* [18] demonstrating decreased mast cell degranulation comparing native and reduced + carboxymethylated bee venom phospholipase is compatible with this possibility. It should be noted, however, that the wasp venom preparation used by us was able to induce the production of both Th1 and Th2 cytokines by lymphocytes from control subjects, indicating that the low amounts of IL-2, IL-4 and IL-5 produced by T cells from allergic subjects were not due to an incapacity of the alkylated antigen to induce Th2 cytokine production. Cytokine profiles of T cell clones are also known to be highly dependent on the culture conditions used to generate the cells, and

conditions used to activate fresh PBMC can also strongly influence the resulting cytokine profiles [29]. Thus, differences in culture conditions, such as antigen dose or availability of costimulatory activities by accessory cells, could explain the low production of Th2 cytokines seen in our study [34,39]. Other explanations are also possible. It is important to realize that in our study, a highly sensitive RT-PCR assay was used to measure cytokine mRNA expression during the first 48 h following antigen-induced activation. In previous studies, cytokine production during the first few days after T cell stimulation was not used to determine cytokine profiles, because cytokine production by PBMC immediately following antigen-induced activation is generally too low to be detected by standard cytokine assays [29]. Rather, the evaluation of T cell clones [11], or uncloned T cells that have been stimulated sequentially with antigens and mitogens or repeatedly stimulated with antigen, has generally been used to induce measurable levels of cytokine production. Furthermore, resting memory T cells must undergo a period of differentiation after stimulation before becoming capable of synthesizing IL-4 [40]. Thus, it is possible that the restricted profile of cytokine mRNAs induced by venom immediately after activation of cells from venom-allergic subjects in this study may subsequently evolve into a typical Th2 profile following repeated stimulation. In this context, IL-2 knockout mice have increased levels of serum IgG1, suggesting that low IL-2 production, as observed by us for cells from venom-allergic subjects, may be permissive for subsequent overproduction of IL-4 [41]. Thus, the initially restricted profile of cytokine mRNAs observed here may prove to be important in the development of Th2 responses. In this case, investigation of the factors controlling the evolution of cytokine profiles may give important insights into the factors predisposing to the development of allergic reactions.

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