

Inflammatory Role of Two Venom Components of Yellow Jackets (*Vespula vulgaris*): A Mast Cell Degranulating Peptide Mastoparan and Phospholipase A1

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Key Words

Inflammatory role · Yellow jackets (*Vespula vulgaris*) · Mastoparan · Phospholipase A1

Abstract

Background: Venom sac extract of yellow jackets *Vespula vulgaris* was toxic in mice when injected intraperitoneally but not toxic when injected subcutaneously. Necropsy showed the toxicity to be an inflammatory response.

Methods: Venom peptide and protein fractions were tested to identify the inflammatory components. The active components were tested to establish whether they might function as adjuvant for venom protein-specific antibody response. **Results:** Venom toxicity required the synergistic action of two venom components, a mast cell degranulating peptide mastoparan and phospholipase A1. Both components stimulated prostaglandin E₂ release from murine peritoneal cells and macrophages. Mastoparan showed a weak activity to enhance IgE and IgG1 responses to a yellow jacket venom protein Ves v 5 in BALB/c mice. It was not possible to assess the adjuvant activity of phospholipase A1 because of its suppression of Ves v 5-specific response. Melittin, a mast cell degranulating peptide from bee venom, was inactive as an adjuvant for Ves v 5-specific response. **Conclusion:**

Yellow jacket venom contains two inflammatory components, mastoparan and phospholipase A1. Our findings suggest that mastoparan can function as a weak adjuvant for TH2 cell-associated antibody response.

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Introduction

Vespid or honey bee venoms are mixtures of proteins, peptides and other small molecules. The proteins are the allergens. These proteins have hyaluronidase, phospholipase A1 or A2 and protease activities and one designated as antigen 5 is of unknown biologic function [1–3]. The structures of these allergens, with the exception of the protease, are known either directly or indirectly. A common feature of these allergens is their partial sequence identity with proteins from other sources.

Venom peptides from several vespids and honey bee have been isolated. They are not allergens with the exception of the honey bee peptide melittin. They have mast cell degranulating (MCD), chemotactic, kinin and neurotoxic activities and some of these activities can lead to recruitment, activation and/or release of mediators from mast cells, neutrophils and other cells and to hypothermia [1, 4–9]. These mediators include cytokines and others;

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Table 1. MCD peptides in bee and vespid venoms

Species	Name of peptide	Sequence ^{1,2}
<i>Vespa analis</i>	mastoparan-A	IKWKA ILDAV KKVI
<i>Vespa basalis</i>	mastoparan-B	LKLKS IVSWA KKVL
<i>Vespa crabro</i>	mastoparan-C	LNLKA LLAVA KKIL
<i>Vespa mandarinia</i>	mastoparan-M	INLKA IAALA KKLL
<i>Vespa orientalis</i>	mastoparan-II	INLKA IAALV KKVL
<i>Vespa tropica</i>	mastoparan-T	INLKA IAAFA KKLL
<i>Vespula lewisii</i>	mastoparan-L	INLKA LAALA KKIL
<i>Vespula vulgaris</i>	mastoparan-V1	INWKK IKS II KAAMN
<i>Vespula vulgaris</i>	mastoparan-V2	INWKK IKS LI KAAMS
<i>Pol. jadwigae</i>	mastoparan-J	VDWKK IGQHI LSVL
<i>Apis mellifera</i>	melittin	GIGAV LKVL TGLPA LISWI KRKRQ Q
<i>Apis mellifera</i>	MCD	IKCNC KRHVI KPHIC RKICG KN

¹ All peptides have C-terminal amides. Their sequences are from references 4–9 with the exception of mastoparan-V2 which is from the present work.

² The sequence differences of mastoparan-V1 and -V2 from the present work are shown in bold-face characters.

some of them can influence the antibody responses of different isotypes.

Honey bee venom has two peptides with MCD activities, melittin and the other known as MCD peptide. Vespid venom has one such peptide mastoparan. These peptides at moderate concentrations have been shown to cause cell lysis. They all are basic peptides with 26, 22 and 14–15 residues, respectively, and their sequences are given in table 1. Mastoparan is reported to be immunogenic for IgG1 in mice [10]. Melittin is immunogenic for IgE and IgG1 responses in mice [11, 12]. It was also reported that melittin functioned as an adjuvant to promote antigen-specific IgE response [11]. The majority of bee-allergic patients had low levels of melittin-specific IgE yet these patients did not have IgE specific for another 18-residue bee peptide, apamin [13].

In this paper we will report our observations on the biological properties of the venom peptides and proteins from yellow jacket *Vespula vulgaris* and honey bee *Apis mellifera*.

Material and Methods

Separation of Venom Components

Venom sac extracts of *V. vulgaris* were from Vespa Laboratories, Spring Mills, Pa., USA. Venom amounts given by Vespa were determined by a colorimetric protein assay. About 45 mg of venom was separated on a 50 × 0.9 cm column of Sephadex G-50 with 0.05 *N* acetic acid as the solvent at a flow rate of 16 ml/h (fig. 1).

The protein fractions IA and IB were immediately neutralized with equal volumes of 0.05 *M* sodium phosphate (pH 5.8) then con-

centrated and equilibrated with the phosphate buffer by ultrafiltration through Amicon membrane. Phospholipase A1 was isolated from protein fraction IB as described previously [14]; its activity was assayed with a synthetic substrate, 1,2-bis(heptanoylthio)-glycerophosphocholine (Cayman Chemical, Ann Arbor, Mich., USA) [15].

The peptide fraction II was desalted on a 2 × 1.6 cm column of C18 silica (Vydac, Hesperia, Calif., USA) and the bound peptides were eluted with 40% 2-propanol in 0.01% trifluoroacetic acid followed by lyophilization. Peptides were separated by reversed phase chromatography on a 5 × 0.9 cm column of C18 silica with a 200-ml gradient of 0–60% 2-propanol in 0.05% trifluoroacetic acid at a flow rate of 60 ml/h. Selected peptides were identified by Edman degradation and mass spectrometry.

Melittin and phospholipase A2 from honeybee venom were isolated as described [13].

Stimulation of Mediator Release from Murine J774

Macrophages and Peritoneal Cells

J774 macrophage cell line was a gift from Dr. Ralph Steinman of this University. Peritoneal cells were collected as described [16]. R10 culture medium contained RPMI-1640, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 56 µM 2-mercaptoethanol and 0.01 *M* HEPES (pH 7.3).

Cells were grown in R10 culture medium at 37 °C and 5% CO₂ for 24 h; 24-well cell culture plates were used. Collected culture fluids were stored frozen until analysis. Cytokines and prostaglandin E₂ were analyzed using kits from R&D Systems (Minneapolis, Minn., USA).

Antibody Response

Groups of 3–4 mice of Balb/c byj strain (Jackson Laboratory, Bar Harbor, Me., USA) were immunized biweekly intraperitoneally, or subcutaneously, with 0.2 or 0.1 ml of phosphate-buffered saline containing 4 µg Ves v 5 and 0.1–1 mg alum together with additional component(s), as indicated in figure 3. Sera were collected on weeks 5 and 7 for analysis of Ves v 5-specific IgG1 and IgE and total IgE. Recombinant Ves v 5 from expression in yeast was used [17].

Ves v 5-specific IgG1 was measured by ELISA as described previously and antibody concentration was determined by comparison with an immuno-affinity-purified sample [17].

Ves v 5-specific IgE was determined by an amplified capture assay [18]. Wells of a micro-titer plate were coated with 10 µg/ml IgE-specific monoclonal antibody 6HD5 [19]. Bound IgE was detected with the sequential binding of the following reagents: 10 µg/ml biotinylated Ves v 5, 4 µg/ml avidin-peroxidase (Sigma), 5 µM biotinyl-tyramine + 0.01% H₂O₂, 4 µg/ml avidin-peroxidase (Sigma) and finally substrate. Concentrations of specific IgE were determined by comparison with melittin-specific monoclonal IgE [12] in the concentration range of 0.01–50 ng/ml, and concentrations of specific IgE are expressed in arbitrary units/ml. Melittin-specific IgE was detected by substitution of the first and second sequential reagents with 1/100 diluted goat anti-rabbit IgG-peroxidase conjugate (Sigma) and 4 µg/ml avidin-peroxidase.

The reproducibility of the above measurements was ± 20%.

Statistical Analysis

This is based on the natural partial ordering of multivariate data [20] to score overall mediator release and antibody response. To simplify analysis, marginal likelihood scores are replaced by scores based on u statistics, modifying Gehan's method for censored data to more general multivariate ordinal data. In essence, each subject is scored by counting the number of observations that are smaller (smaller or equal in all variables and smaller in at least one variable) and subtracts the number of observations that are larger. For univariate observations, this procedure yields ranks.

Scores for mediator release and antibody response are correlated by means of Spearman's rank correlation, with p values determined from the t-distribution. To compare between groups after stratifying for possible cofounders, a stratified Wilcoxon test is employed [21], again using scores based on u statistics to allow for multivariate data. All p values are given on a per-comparison basis.

Results

Separation of *V. vulgaris* Venom Components

Venom sac extract was fractionated by Sephadex G-50 filtration into protein fractions IA and B, and peptide fraction II (fig. 1a). SDS gel electrophoresis showed protein fraction IB to contain hyaluronidase, phospholipase A1 and antigen 5 (data not shown). Reversed phase column chromatography of peptide fraction II led to the isolation of its major component (fig. 1b). Sequence analysis showed the major component to have the sequence shown in table 1 with a calculated molecular weight of 1,731.5. Synthetic and natural mastoparans showed indistinguishable mass units of 1,731.5 and 1,731.1, respectively. Assuming 80% recovery, the estimated mastoparan content was 7.9 mg for 100 mg of venom sac extract.

Mastoparan from *V. vulgaris* has been previously characterized [7]. As seen in table 1, the present sequence differs from the reported one at residues 9 and 15.

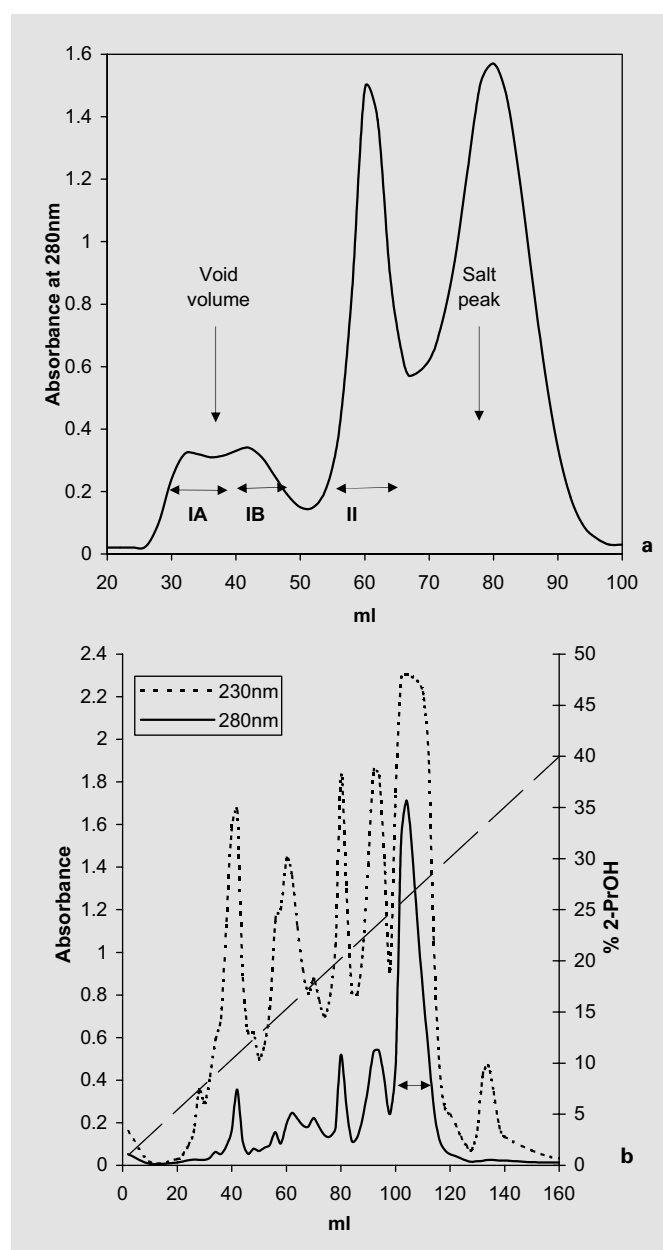


Fig. 1. **a** Gel filtration of *V. vulgaris* venom sac extract to yield peptide and protein fractions. **b** Isolation of mastoparan from peptide fraction II by reversed phase chromatography. See Materials and Methods for details.

Mastoparan is reported to facilitate the activity of honey bee phospholipase A2 with a long chain fatty acid phosphatidyl choline as substrate [22]. We measured the activity of *V. vulgaris* phospholipase A1 with a short chain fatty acid phosphatidyl-choline as substrate alone or in the presence of 2.5×10^5 molar excess of mastoparan and no

Table 2. Toxicity of venom components in mice

Group	Venom		Route of injection	Mice dead on or before		
	amount	component		day 1	day 4	day 7
1	0.8 mg	protein Ib + peptide II	i.p.	1	1	1
2	1.6 mg	as above	i.p.	3		
3	0.8 mg	as above	s.c.	0	0	0
4	1.6 mg	as above	s.c.	0	0	0
5	0.8 mg	protein Ib + mastoparan	i.p.	0	0	0
6	1.6 mg	as above	i.p.	3		
7	0.8 mg	PLA1 + peptide II	i.p.	2	1	
8	1.6 mg	as above	i.p.	3		
9	0.8 mg	PLA1 + mastoparan	i.p.	0	0	0
10	1.6 mg	as above	i.p.	3		

Groups of 3 BALB/c byi mice were each injected intraperitoneally or subcutaneously with 0.2 ml of venom in phosphate-buffered saline. The component weights are expressed in venom equivalent amounts; 1 mg of venom sac extract was estimated to contain 0.04 and 0.08 mg of phospholipase A1 (PLA1) and mastoparan, respectively.

difference in activity was observed. Similarly, we did not observe any change in the activity of honey bee phospholipase A2 in the presence of mastoparan.

Toxicity of Venom Components in Mice

Mice died within 1 week on intraperitoneal injections of 0.4 mg *V. vulgaris* venom sac extract. Necropsy of venom-injected mice showed severe peritonitis with influx of lymphocytes, macrophages and neutrophils. No significant lesions were observed in other organs and tissues.

Comparing groups 1 and 2 with groups 3 and 4 in table 2 showed toxicity of admixture of protein and peptide fractions, Ib and II when injected intraperitoneally but not subcutaneously ($p < 0.01$, stratified for amount). Thus the following results are given for the intraperitoneal route only.

Mastoparan showed a clear dose-response relationship when given in combination with protein fraction Ib (group 5 versus 6, $p = 0.025$, exact) or phospholipase A1 (group 9 versus 10, $p = 0.025$, exact).

At low dose, peptide fraction II was more lethal than mastoparan when given in combination with protein fraction Ib (group 1 versus 5), or with phospholipase A1 (group 7 versus 9), with $p < 0.01$, stratified for co-administration.

Other results not given showed similar injections of equivalent amounts of the peptide or protein fraction alone did not cause death. Therefore the findings in table 2 together indicate the lethal action of venom re-

quires the synergistic action of phospholipase A1, mastoparan and possibly other peptide components.

Venom Component Induced Mediator Release

The data from one set of 24-hour mouse macrophage J744 cultures are given in figure 2. Data from another set of experiments (not shown) are within $\pm 15\%$ of those in figure 2. The data for wells 1–3 suggest that mastoparan stimulated the release of the lipid mediator prostaglandin E_2 and the cytokine TNF- α . Phospholipase A1 alone and in combination with mastoparan showed a dose-dependent stimulation of prostaglandin E_2 (groups 4–7, $p < 0.01$), and it showed an inhibitory effect on the release of TNF- α ($p < 0.01$).

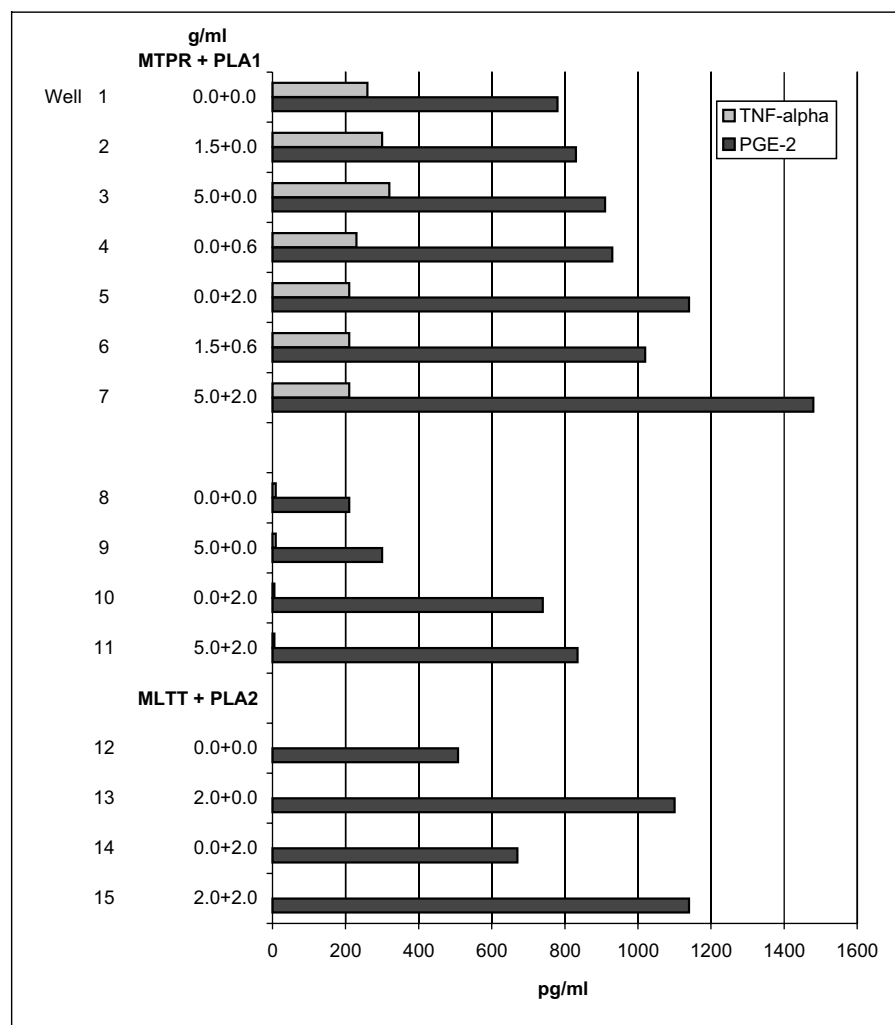
Mastoparan and phospholipase A1 stimulated the release of prostaglandin E_2 from mouse peritoneal cells (wells 8–11). But no significant change was observed in the secretion of TNF- α when compared to controls.

Melittin and phospholipase A2 were shown to stimulate the release of prostaglandin E_2 from J744 cells (wells 12–15); release of TNF- α was not tested.

Testing Adjuvant Activity of Venom Components

We used yellow jacket antigen 5, Ves v 5 as a model immunogen. Mice were immunized with Ves v 5 and alum, with or without added venom components. Groups 1–6 and 7–17 mice were immunized by the subcutaneous and intraperitoneal route respectively (fig. 3). Similar antibody responses at weeks 5 and 7 were observed; only those at week 7 are given.

Fig. 2. Venom component stimulated release of mediators from mouse J744 macrophages and peritoneal cells. Wells 1–7 and 12–15 contained J744 cells and wells 8–11 contained peritoneal cells. Concentrations of mastoparan (MTPR), mellitin (MLTT) and phospholipases A1 and A2 (PLA1 and 2) in culture are shown along the y-axis.



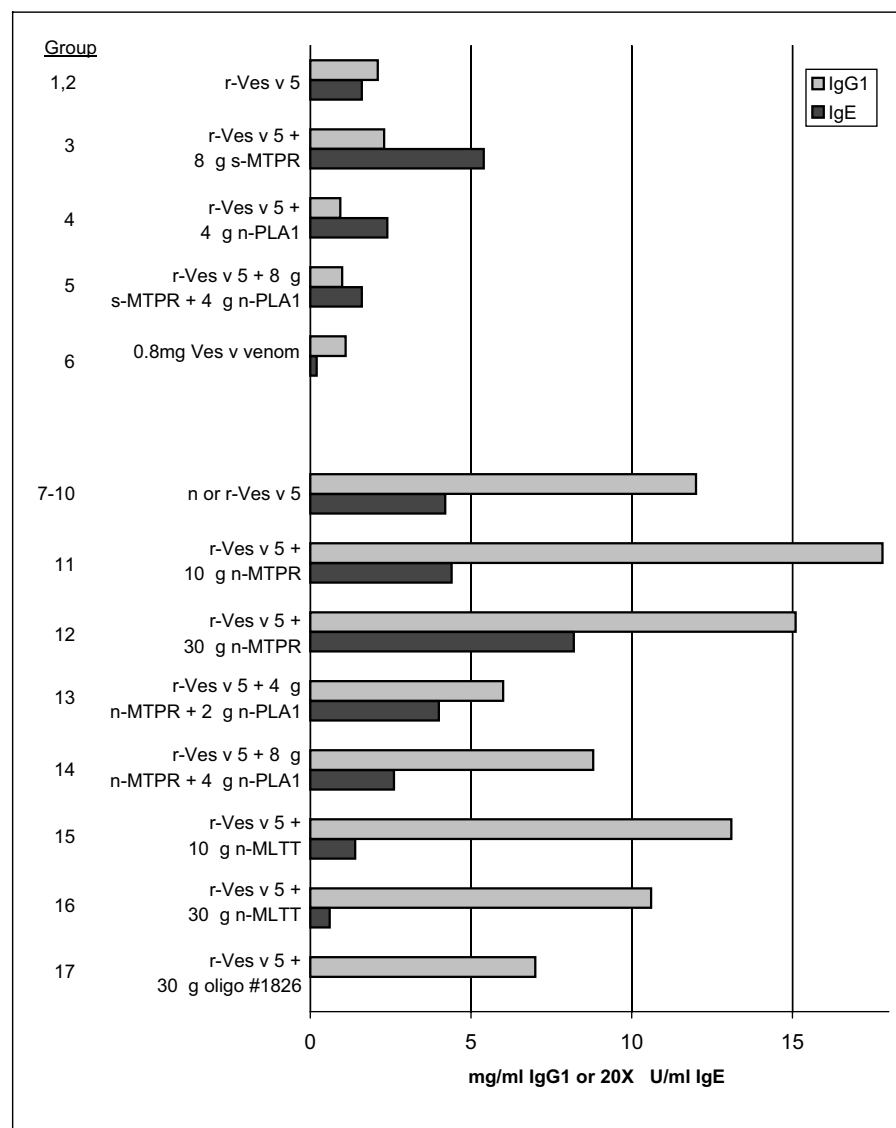
Groups 1–2 are control mice immunized subcutaneously with Ves v 5; their average antibody data are shown in figure 3 and data for individual groups are given in the figure legend. Group 3 mice were immunized with Ves v 5 + mastoparan. Their specific IgE and IgG1 levels were, respectively, 338 and 109% of the control mice. Groups 4 and 5 mice were immunized with Ves v 5 + phospholipase, and Ves v 5 + mastoparan + phospholipase, respectively. Their specific IgE and IgG1 levels were both about 125 and 46% of the control mice. Group 6 mice immunized with Ves v venom also showed decreased antibody levels.

Groups 7–10 mice are control mice immunized by the intraperitoneal route and their average antibody titer is shown in figure 3 (data for individual groups in figure legend). Ves v 5-specific IgE and IgG1 responses of test groups 11 and 12 at two different doses of mastoparan

were respectively 105–195 and 126–148% of the control groups 7–10. Those of test groups 13 and 14 at two different doses of mastoparan + phospholipase were 62–95 and 50–73% of the control. Groups 7–14 mice immunized intraperitoneally consistently showed higher antibody levels than their corresponding groups 1–5 immunized subcutaneously did.

The results of groups 11 and 12 mice suggest a dose-response relationship of mastoparan and Ves v 5-specific IgG1 and IgE responses ($p < 0.06$). The results of group 3 by the subcutaneous route are compatible with those of groups 11 and 12 by the intraperitoneal route. By assuming the dose effect to be minor, the results of these three groups can be pooled. Aggregating the results stratified for the route of immunization shows evidence that mastoparan has an adjuvant effect for Ves v 5-specific IgG1 and IgE responses ($p < 0.05$).

Fig. 3. Antibody responses to Ves v 5 in the presence of added venom components. Mice groups 1–6 and 7–17 were immunized by the subcutaneous or intraperitoneal route, respectively. Prefixes n, r and s denote natural, recombinant and synthetic, respectively. Average antibody levels of groups 1–2 and 7–10 are given. Specific IgE levels of groups 1–2 are 0.07 and 0.08 μ U/ml and their specific IgG1 levels are 1.5 and 2.7 mg/ml. Specific IgE levels of groups 7–10 were 0.28, 0.14, 0.29 and 0.14 μ U/ml and their specific IgG1 levels were 14.0, 8.4, 10.8 and 14.8 mg/ml.



The results of group 4 mice suggest that phospholipase suppressed Ves v 5-specific responses. The suppressive effect of phospholipase was also observed with the phospholipase + mastoparan test groups 5, 13 and 14, and with group 6 mice immunized with venom that contains Ves v 5, phospholipase and other proteins. Phospholipase A1 is immunogenic [14]; antibody response to phospholipase was not measured in this work.

Groups 15 and 16 mice were used to test melittin at two dose levels as an adjuvant for Ves v 5-specific response. Their specific IgE and IgG1 levels were respectively within 38–117 and 88–109% of the controls.

CpG oligodeoxynucleotide is known to stimulate Th1-dominated antibody responses [23]. It was used as a con-

trol adjuvant in group 17 mice. In accord with the known property of this adjuvant, their specific IgE and IgG1 levels were reduced to 0.09 and 58% of the control and there was a marked rise of their specific IgG2a level (data not shown).

Discussion

We observed yellow jacket venom to be lethal in mice when injected intraperitoneally but not subcutaneously. The lethal action is an inflammatory response at the site of venom injection as evidenced by the necropsy data. Further studies in table 2 showed the lethal action re-

quires the combined action of one peptide and one protein component, mastoparan and phospholipase A1.

Mastoparan was discovered for its activity to induce release of histamine and other mediators from mast cells [4]. It binds to cell membranes [24, 25] and it can act as a strong secretagogue for different cell types. For example, mastoparan is reported to stimulate rapid release of the inflammatory mediators, TNF- α , IL-1 β and nitrous oxide into peritoneal exudates after injection at a dose of 10 μ g/mouse [26], and it is also reported to stimulate the release of prostaglandin E₂ from rabbit astrocytes [27]. These mediator releases are related to its diverse range of biochemical activities. They include stimulation of phospholipases A2 [22], C [28] and D [29, 30] and G-protein activation [31].

We did not detect mastoparan stimulation of phospholipase A1 or A2. The reported stimulation of phospholipase A2 activity was made with a long chain fatty acid phosphatidyl choline as substrate [22], while we used a short chain fatty acid substrate. Thus the different findings may be due to the nature of substrates.

Phospholipases and a phospholipase-activating protein, as well as melittin, stimulated the release of prostaglandin E₂ from macrophages [32]; melittin has sequence homology with the phospholipase-activating protein. The action of melittin had also been suggested to its activation of phospholipase A2.

Our data in figure 2 show that mastoparan and phospholipase A1 each independently induced the release of prostaglandin E₂ from cultured mouse macrophages or peritoneal cells. Phospholipase A1 was >1,000-fold more active than mastoparan was on a molar basis, since its action leads to an increased pool of prostaglandin precursor. We did not detect significant release of TNF- α . The synergistic lethal action of mastoparan and phospholipase A1 may be due to increased levels of prostaglandin E₂ and possibly other inflammatory mediators that we have not measured.

Prostaglandin E₂ is a mediator of inflammation as well as a modulator of immunity [33]. It acts on antigen-presenting cells to promote the expression of IL-10 and to inhibit the expression of IL-12. It acts on T cells to enhance production of TH2 cell-associated cytokines and to inhibit their production of TH1 cell-associated cytokines. And it acts in concert with IL-4 to promote IgG1 and IgE switching in B cells. In numerous clinical conditions there is a correlation between elevated IgE levels and overproduction of prostaglandin E₂.

Bee venom phospholipase A2 is reported to induce mediator release and IL-4 synthesis in rat basophils [34].

An enzymatically inactive recombinant phospholipase A2 that differs from the natural enzyme by a single residue at its active site was found to be inactive in mediator release or IL-4 synthesis. Phospholipase A1 is likely to have a similar role as phospholipase A2 has because of their similar enzymatic specificity. Phospholipase A1 catalyzes the hydrolysis of acyl residues at positions 1 and 2 of phospholipid and phospholipase A2 catalyzes only the acyl residue at position 2.

The above discussions indicate that mastoparan/melittin and phospholipase A1/A2 can induce the release of TH2 cell-associated mediators from basophils/mast cells, macrophages and possibly other antigen-presenting cells. Hence they may function as an adjuvant to promote IgG1 and IgE responses. The data in figure 3 show that mastoparan has a weak activity to enhance IgG1 and IgE responses to yellow jacket antigen 5 in mice ($p < 0.05$). This is to be compared with a TH1 cell-associated adjuvant CpG oligo #1826 in figure 3 that showed a modest suppression of IgG1 but a marked suppression of IgE.

No adjuvant activity was detected for phospholipase A1 as it showed a suppressive effect on Ves v 5-specific antibody response. This may be due to antigenic competition as phospholipase is immunogenic.

Mellitin was not found to have adjuvant activity for Ves v 5-specific antibody response, although melittin like mastoparan stimulated the release of prostaglandin E₂ from macrophages. Others found mellitin to be an adjuvant for ovalbumin-specific IgE response in mice [11]. The two different findings on mellitin may reflect the experimental conditions used, as IgE responses in mice are antigen, dose and mice strain dependent [35, 36]. For example, the bee venom phospholipase A2 stimulated a high IgE response in low doses (0.1 μ g/injection) and a low response in high doses (10 μ g), but the inactive mutant stimulated a low IgE response in high or low doses [34].

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