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Mast Cell Activation by Hornet (*Vespa orientalis*) Venom¹

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Abstract. As far as its effect on the activation of rat peritoneal mast cells is concerned, the venom of young hornets (age 0–24 h) differs from that of adult hornets (over 24 h of age). ‘Juvenile’ venom caused activation mainly in rats previously sensitized to it, whereas ‘adult’ venom induced activation regardless of presensitization. Furthermore high concentrations of ‘adult’ venom were cytotoxic to the entire murine peritoneal cell population, but ‘juvenile’ venom was not toxic. SDS-PAGE data demonstrate that the venom of young hornets contains a unique component (82 kD), nonexistent in adult venom. Our data demonstrate that hornet venom activity is correlated with the hornet’s age.

Introduction

The oriental hornet, *Vespa orientalis* (Hymenoptera, Vespinae), is a social insect widespread throughout the Mediterranean area [1]. Hornet venom causes many severe allergic and toxic effects in mammals [2–5]. Biochemical and physiological investigations have demonstrated that hornet venom contains numerous different proteins with various biological activities [6, 7]. The major proteins are enzymes such as hyaluronidase, phospholipase and some proteases. In addition the venom is known to contain low molecular weight substances such as biogenic vasoamines (acetylcholine, histamine, serotonin) and peptides (kinins and also mastoparans, the mastoparans are mast cell-degranulating factors [8, 9]).

It is documented that the activity of hornet venom on mice [10] and tissues [11] is dependent on the hornet’s age. Thus the LD₅₀ of young hornet venom in mice was found to be about 500 mg/kg mouse, whereas adult hornet venom was ten times more active (LD₅₀ = 50 mg/kg mouse). In this connection, we should mention that we have previously defined a young hornet as one whose age is less than 24 h (from

eclosion), while an adult hornet is one older than 24 h [10]. Hornet sting toxicity and allergy represent one of the major hazards to field workers as well as several other occupations in the Middle East. It is therefore our aim to find antigens which do not cause toxic reactions and may be used for clinical treatment. Our present report focuses on the biological effects of the venom at the cellular level. For this purpose we have obtained from rats a whole peritoneal cell wash which contains mast cells, macrophages, and some lymphocytes.

Materials and Methods

Venom Extraction

Nests of *V. orientalis* were sought for in the Tel-Aviv metropolitan area and, once detected, they were dug up and transported to our laboratory [12]. The entire adult hornet population was removed from the combs and the latter were then placed in special breeding boxes. Eclosing young hornets were collected every 24 h, so that we could obtain two hornet populations: (a) hornets up to 24 h of age, designated *V. orientalis* young (Voy), and (b) hornets older than 24 h, designated *V. orientalis* adult (Voa). The newly eclosed hornets (and wasps) markedly differ from the older ones by two signs: (1) the color of the youngster’s cuticle is bright while that of the older one is darker, and (2) the four wings of the newly eclosed hornets are not folded lengthwise. They do not fold lengthwise until the hornets are 18–24 h past eclosion and will always be folded lengthwise when the insects are at rest. All collected specimens were kept in deep-freeze at –30°C. A few minutes prior to

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use, the hornets were thawed out and venom was extracted by eversion of the sting, through a plastic membrane to avoid contamination by other possible secretions, pressure on the abdomen and collection of the venom drop which appeared on the tip of the stinger. This method, albeit slow and demanding, is in our opinion the best for obtaining pure venom drops, yielding about 2–3 μ l of clear venom from each hornet.

The protein concentration in the venom from both age groups was determined by the method of Lowry et al. [13], and proved to be about 150 mg protein/ml Voa venom as compared to about 40 mg protein/ml Voy venom, meaning that the protein concentration in adult hornet venom was 3- to 4-fold greater than in young hornet venom.

Antigen Preparation

The venom, which also contains the proteins comprising the antigen material (Ag) was used in two ways: (a) for primary immunization (as a 1st Ag) in vivo, wherein 2 μ l of venom were dissolved in 1 ml of saline (0.9% NaCl) for each injected rat, and (b) for incubation (as a 2nd Ag) in vitro, wherein a series of seven venom dilutions in 0.1% bovine serum albumin plus phosphate-buffered saline was carried out, yielding venom concentrations between $1:10^3$ and $1:10^6$.

Rat Peritoneal Cell Wash

Wistar rats, age 3 months, were injected intraperitoneally with either Voy or Voa venom. The control group was injected with saline only. The 3 rat groups (2 experimental and 1 control) were comprised of 5 rats each, and each experiment was run in triplicate. In all, 45 rats were used. Forty days after injection with the primary Ag, the rats were sacrificed by CO₂ chamber anesthesia and harvested for peritoneal cells by rinsing the peritoneal cavity with 0.1% bovine serum albumin in phosphate-buffered saline. Following this, the collected cell suspension was centrifuged for 10 min at 300 g (5°C). The supernatant was decanted to leave a pellet of peritoneal cells.

Mast Cell Secretion Dye-Binding Exclusion Method

To the series of Voa and Voy venom dilutions (the 2nd Ag) were added peritoneal cells from each of the test groups to finally yield 8 assessment groups, as detailed in table 1. After incubating cells and venom for 15 min at 37°C, the reaction was terminated by rapid centrifugation in the cold and aliquots were pipetted onto glass slides, stained in toluidine blue + 80% alcohol for 20 min and rinsed in 70% alcohol. Upon drying, the aliquot samples were scanned for intact mast and all other cells and compared with the control samples (groups 3 and 7 in table 1) by the dye-exclusion method [14]. The percent of cells activated as compared to the control group was defined as a mast cell activation unit.

Electron Microscopy

The rat peritoneal cell suspension was centrifuged and the pellet was immediately fixed for 2 h in a cold solution of 3.2% glutaraldehyde and 1.6% formaldehyde in 0.1 M sodium cacodylate and post-fixed in a solution of 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The fixed cells were dehydrated in ethanol, washed twice with propylene oxide and embedded in Poly Bed 812 kit (Poly-Science, USA). Ultrathin sections, post-stained with uranyl acetate and lead citrate, were observed using a JEOL 100B Transmission Electron Microscope (JEOL, Japan).

Table 1. Order of treatment with venom in the rat groups

		Group No.							
		1	2	3	4	5	6	7	8
1st Ag	Voy	Voy	Voy	saline	Voa	Voa	Voa	saline	
2nd Ag	Voy	Voa	saline	Voy	Voa	Voy	saline	Voa	

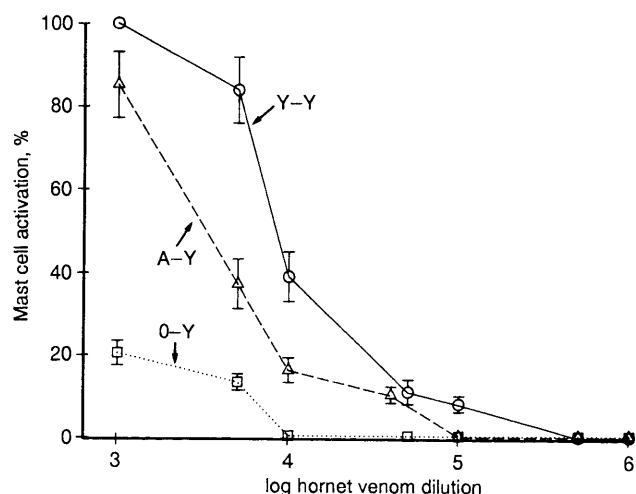


Fig. 1. Quantitative analysis of mast cell activation following incubation with young hornet venom. The first letter (in Y-Y, A-Y and O-Y) indicates the primary immunization: Y = young hornet venom; A = adult hornet venom; O = saline as control. The second letter (Y) indicates that young hornet venom was used as a secondary antigen. Each data point is the mean $\pm$ standard deviation of 5 rats. The significance of the decline, at high concentration, was $p < 0.01$.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Voy and Voa venoms were compared by SDS-PAGE after the method of Laemmli [15]. The venom drops from a mixture of 25 hornets were mixed with sample buffer (2% SDS, 5% 2-mercaptoethanol), boiled for 5 min and separated on a 5% stacking and 10 or 12.5% separating SDS-PAGE. The separated proteins were stained with Coomassie blue R-250.

Results

Mast Cell Activation under the Influence of Voy Venom

As seen in figure 1, there were significant differences in the effect on murine mast cells between Voy venom, as 2nd Ag in rats pre-sensitized with either

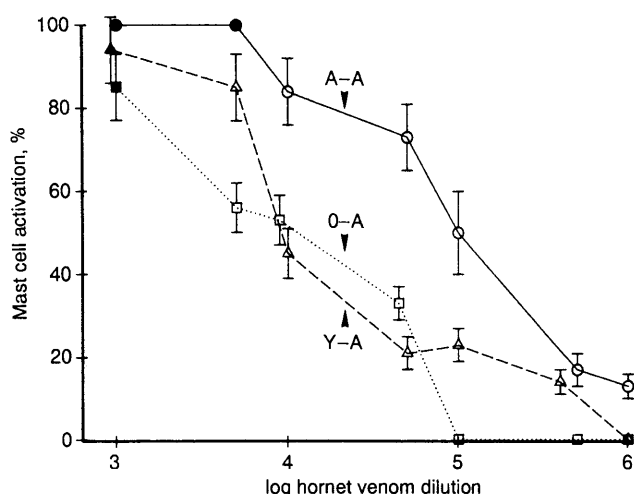


Fig. 2. Quantitative analysis of mast cell activation following incubation with adult hornet venom. The dark symbols indicate nonspecific cytotoxicity. The first letter (in A-A, Y-A and O-A) indicates the primary immunization: A = adult hornet venom; Y = young hornet venom; O = saline as control. Each data point is the mean $\pm$ standard deviation of 5 rats. The significance of the decline, at high concentration, was $p < 0.01$.

Voy or Voa venom, and Voy venom, as 2nd Ag, in control rats injected merely with saline. Furthermore, it can be seen that with maximal venom concentration the mast cell activation was 100 and 85% when the 1st Ag was Voy and Voa venom, respectively, whereas the corresponding degranulation with cells from control rats (all of 15 rats) was only 40%. It is important to point out that there were only minor (albeit statistically significant) differences between the activation of mast cells from animals pre-sensitized with Voy venom and that of mast cells from animals pre-sensitized with Voa venom, when the 2nd Ag in both cases was Voy venom. In no concentration of Voy venom and in none of the experimental groups, did we observe a cytotoxic effect on the entire cell population (lymphocytes, macrophages, etc.).

Mast Cell Activation under the Influence of Voa Venom

Only minor (albeit statistically significant) differences were observed in mast cell degranulation with Voa venom as 2nd Ag between rats pre-sensitized with Voa or Voy venom and in control rats injected with saline only. The results are presented graphically

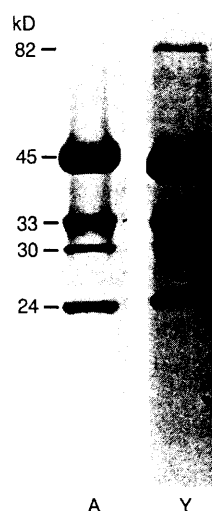


Fig. 3. SDS-PAGE of *V. orientalis* venom. The adult venom (A) concentration is equivalent to 1 hornet while the young venom (Y) is equivalent to 4 hornets. The molecular weights of the main 5 bands are indicated.

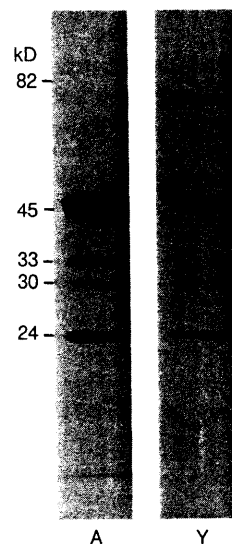


Fig. 4. SDS-PAGE of *V. orientalis* venom as in figure 3. The venom concentration in (A) is one quarter of one venom sac equivalent content, while in (Y) a content of one venom sac is separated.

in figure 2. Our findings here, particularly the curve describing the mast cell activation under Voa venom of cells not previously sensitized by any venom, suggest that a considerable part of the degranulation in those experimental groups, where Voa venom is the

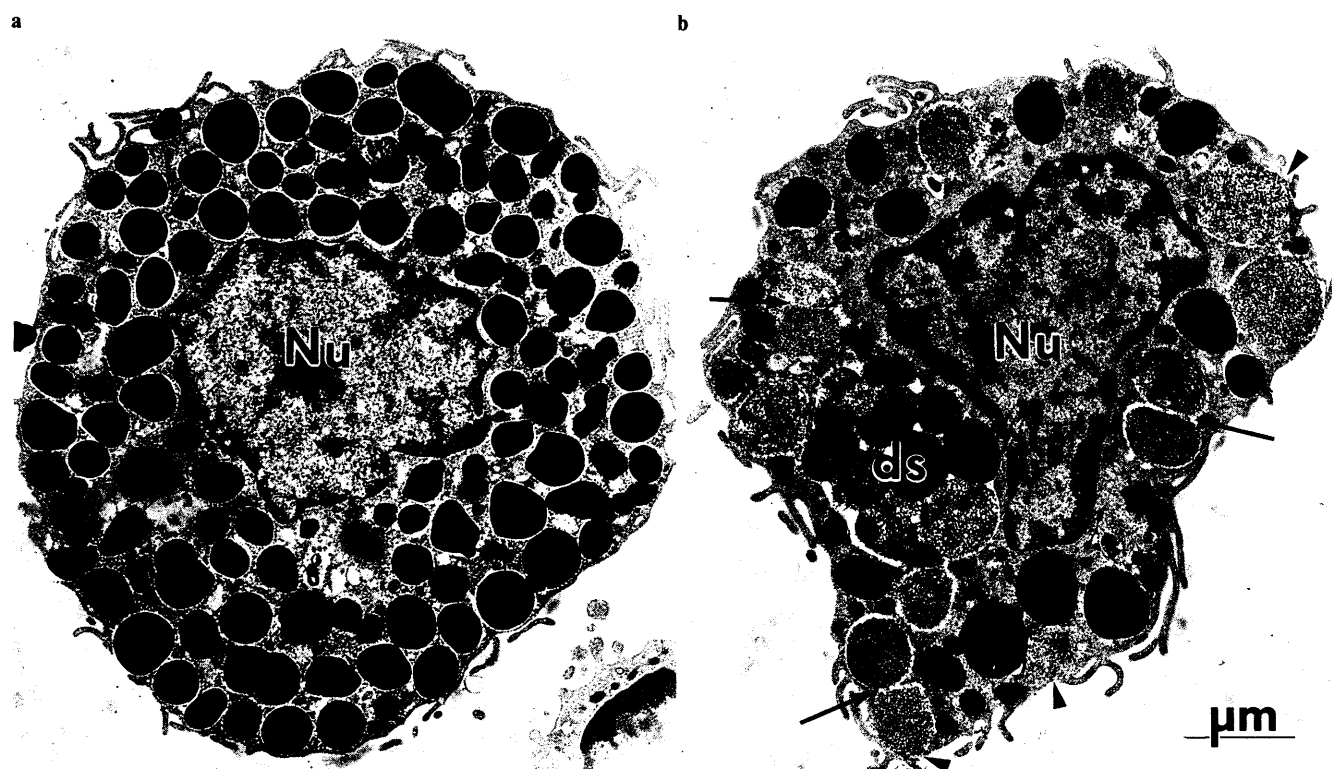


Fig. 5. Electron micrograph of rat peritoneal mast cells before (a) and following degranulation (b) induced by adult hornet venom. In the resting mast cells (a) numerous highly homogenous electron dense granules are observed. In the activated mast cell (b) the granular content is heterogeneous. Nu = Nucleus; ds = degranulation sac; arrow heads indicate the omega-shape characteristics of granule plasma membrane fusion while the arrow indicates granule-granule fusion.

2nd Ag, is attributable to direct activity of components in the venom and not merely to immunologic elements. Here, Voa venom in its high concentrations was also cytotoxic to the entire cell population (fig. 2). It is noteworthy that a comparison of the results shown in figure 2 with those in figure 1 reveals that Voa venom was 10 times more active ($ED_{50} = 1:10^4$) than Voy venom ($ED_{50} = 1:10^5$).

Interestingly, a quasi-rouleau formation of the lymphocytes was observed at a high venom concentration independent of the hornet's age.

Venom Separation in SDS-PAGE

Comparing Voa and Voy venom by SDS-Page revealed that the main protein fractions which were recognized in Voa venom, namely hyaluronidase (45 kD), phospholipase (30, 33 kD) and antigen 5 (24 kD), also occur in Voy venom judging by the molecular weight. In Voy venom, however, another frac-

tion with a molecular weight of 82 kD and not detected in Voa venom was definitely recognized. The pertinent comparison is made in figure 3. It should be pointed out that this 82-kD band shows up in Voy venom even when the quantity of venom in the fractionation is very low (i.e., at the limit of sensitivity of this system) as can be seen in figure 4.

Electron Microscopy Findings

Normal homogeneous electron-dense granules were found in mast cells of the control rats (fig. 5a). In contrast, in the mast cells activated by Voa venom at a dilution of $1:10^5$ (ED_{50}), the granular substructures were highly heterogeneous in the following ways: (a) in many granules the electron-dense contents were interspersed with regions of low density; (b) in most instances the omega-shape characteristics of granule extrusion were observed, and (c) degranulation sacs were detected.

Discussion

Mast cells, which play a pivotal role in anaphylactic shock by releasing histamine and producing prostaglandins as well as leukotrienes, served in the present study as a bioassay for the discrimination between the immunologic and toxicologic effects of oriental hornet venom. The findings of the present investigation show that the toxic and immune effects of hornet venom are separable to a large extent by the use of two types of venom, namely the venom of young hornets (voy) as compared to that of adult hornets (Voa). Thus, high concentrations of Voa venom ($1:10^3$, $5:10^3$) elicited high cytotoxicity (85–100% mortality) of mast and other peritoneal cells examined from rats, whereas no concentration of Voy venom, even such as elicited 100% activation of mast cells, proved cytotoxic to murine peritoneal cells. There was a significant difference between these two venom types also insofar as the ED_{50} of mast cell activation is concerned. Thus, whereas with Voa venom the ED_{50} was $1:10^5$, with Voy venom it was $1:10^4$, and this in rat groups presensitized by venom. In the control groups (i.e. in cells from rats not presensitized by any sort of venom) the difference was even greater: the Voa venom ED_{50} was $1:10^5$, while the Voy venom ED_{50} was $>1:10^3$. Even if we take into account the fact that the protein concentration in Voa venom is 3–4 times greater than in Voy venom, the differences in the ED_{50} are still large and significant. We should point out that our measurement of mast cell activation was made microscopically and not biochemically because the venom itself contains high concentrations of vasoamines [16, 17], significantly above the peritoneal mast cell content, therefore the quantitation on the basis of secretion becomes inaccurate. Biochemical estimation of secreted enzyme content was similar to the microscopic data. However, 5 times more cells were needed to quantify cell activation by the biochemical methods. To assess the nature of mast cell activation, we used electron microscope techniques to support the idea that the observed activation is due to mast cell degranulation.

Our finding that the control group yielded the greatest difference in the rate of mast cell activation suggests that, whereas Voa venom contains substances directly affecting activation, without recruitment of the immune system, Voy venom contains only a few substances with such a direct effect. In other words, whereas Voa venom exerts an immune

as well as a toxic effect, Voy venom produces primarily an immune effect, possibly through the antigen antibody mechanisms. Immunochemical and biological studies of the venom from young and adult hornets demonstrate that the venom from young insects provokes more attenuated cutaneous and systemic reactions than those elicited by adult insect venom [18, 19].

As a preliminary step in differentiating between the components of Voa and Voy venom, we resorted to SDS-PAGE. This separation procedure yielded a similar picture of the protein makeup in both venom types as for molecular weight, except for one extraneous fraction in Voy venom which showed up clearly at a molecular weight of 82 kD. In this connection, it should be noted that Rosenberg et al. [11] examined phospholipase A_2 activity in oriental hornet venom by measuring the amount of free fatty acids released from egg yolk following its incubation with venom from hornets at various ages. In that study it was found that in freshly eclosed hornets (age 0) the amount of free fatty acids in the incubation mixture was near zero, whereas in hornets aged more than 1 day the amount of free fatty acids was considerable. In addition there are other studies which have shown a facilitation of phospholipase A_2 activity by certain peptides in hornet venom which have been designated as mastoparans, and evidence exists to indicate that the release of histamine from human mast cells is dependent on phospholipase. If we collate these findings from the literature with the findings of the present study, we may draw two possible conclusions: either Voy venom lacks the mastoparan needed to activate the phospholipase to act upon cells or that the phospholipase detected in Voy venom by molecular weight (the two bands at 30 and 33 kD) is precursory and undergoes some sort of activation within the first 24 h of hornet life.

Our preliminary studies demonstrate that the high molecular weight proteins alone have no mast cell activation capacity. Judging by the findings of the present study, it would seem that the infliction of a sting by a single hornet to a person of average weight, with a blood volume of about 5×10^3 ml, should not have any systemic effects. If, however, a person not previously stung is attacked and stung by say 5–10 hornets, then one can expect a systemic response of a toxic nature, much as was observed in our control group administered Voa venom. Since in nature only adult hornets sting (because the juvenile hornets do not

leave the nest until they are mature), then for the person who is accidentally stung by hornets the relevant findings are those that we obtained with the control group receiving Voa venom. However, in the case of a person undergoing a regimen of desensitization, where the customary maintenance dosage is 100–200 µg, the possible (and alternative) administration of Voy venom, which is less toxic as judged by our findings (fig. 1), might improve the treatment.

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