Mast Cell Histamine Release Induced by Portuguese Man-of-War (Physalia) Venom

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Abstract

MAST CELL HISTAMINE RELEASE INDUCED BY
PORTUGUESE MAN-OF-WAR (PHYSALIA) VENOM.

by

A. LESTER FLOWERS¹

Richet and Portier studied the immunization of dogs against attenuated toxic extracts of the sea anemone and showed that, when given secondary injections of sublethal amounts of native toxic extracts, the dogs experienced severe pruritus and insensitivity followed by death. They called this increased sensitivity to the toxins, anaphylaxis. Richet and Portier, and later Jacques and Schachter, and Uvnäs also studied the direct pruritic and histamine releasing effects of cnidarian (e.g. Physalia, Actinia, Cyanea) toxic extracts. While the anaphylactic effect, discovered by Richet and Portier, has been extended by numerous studies to provide the foundation of our current knowledge of allergic atopy and anaphylaxis, the study of the direct pruritic or histamine releasing effects of cnidarian toxins have been left largely unstudied.

The present study investigates the general mechanism whereby nematocyst venom causes the direct release of histamine from isolated mast cells. It attempts to compare this effect, histamine release,

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other studies using whole tissue extracts and/or whole animals. Crude peritoneal mast cells were obtained from male Sprague-Dawley rats, washed and suspended in Locke's solution, and purified by sedimentation. To characterize the requirements for Physalia venom-induced histamine release, mast cells and nematocyst venom were incubated together in the presence or absence of calcium, at different temperatures, and with ATP depleted and ATP supplied mast cells. Following treatment the samples were assayed fluormetrically for both released histamine and released lactate dehydrogenase, a cytoplasmic enzyme marker.

Physalia venom causes the release of both histamine and lactate dehydrogenase. The release of histamine is dose-dependent and approaches 100% at high doses of venom. The dose-response curve is hyperbolic in shape. Histamine release is independent of calcium and energy, and the rate of release is optimal in the range from 10-30°C. The release of lactate dehydrogenase is concomitant with histamine release; release is also dose-dependent and is more sensitive to the venom than histamine release. ATP depletion increases the sensitivity of venom-induced lactate dehydrogenase release while having no effect on histamine release.

It is concluded that Physalia venom induces the release of both histamine and lactate dehydrogenase from isolated rat peritoneal mast cells by a cytolytic mechanism. This conclusion is based on the following observations: histamine release is neither dependent on a cellular source of energy nor calcium; histamine release is fairly temperature insensitive; and lactate dehydrogenase is released along with histamine.
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of the Requirements for the Degree Master of Arts
in Biology

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Each person whose signature appears below certifies that this manuscript in his opinion is adequate, in scope and quality, in lieu of a thesis for the degree Master of Arts.

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INTRODUCTION

The Portuguese Man-of-War (*Physalia physalis*), is the most hazardous cnidarian in the Atlantic and Caribbean basins. Next to the Indo-Pacific sea-wasp (*Chironex fleckeri*) it is considered the most toxic and deadly of all stinging marine organisms (1). Stinging symptoms from these cnidarians include intense pain, reddening and swelling of the skin, tightness in breathing, and pulmonary congestion (2).

It is commonly regarded that nematocyst venoms are potent and that their constituent toxins are proteins. While much physiological work has been done at the systemic and organ level little is known of the cellular and molecular mechanisms of action of nematocyst venoms, much less of their constituent toxins. Consequently, it has not been possible to determine the cellular basis of toxicity. However, several of the commonly observed symptoms may be explained by the involvement of mast cells. Portier and Richet (3) first described anaphylaxis using extracts of *Physalia* and *Actinia* tentacles. Their initial findings were extended by Jaques and Schachter (4) using perfused cats skin and Uvnäs (5) using isolated rat mast cells. These studies, however, were performed using whole tissue, alcoholic extracts, and, thus, it is not known whether the observed effects are due to constituents of the nematocysts.

The present study extends these earlier findings concerning cnidarian toxin-induced histamine release in two ways: (i) by using venom obtained directly from isolated *Physalia* nematocysts, and (ii) by
describing the general mechanism by which the nematocyst venom causes histamine release from isolated rat peritoneal mast cells.

MATERIALS AND METHODS

EGTA (Ethylene glycol-bis-$\beta$-aminoethyl ether-N,N'-tetraacetic acid), HEPES (N-2 hydroxyethyl piperazine-N-2-ethanesulfonic acid), $\beta$-nicotinamide adenine dinucleotide (Grade III), $\beta$-nicotinamide adenine dinucleotide, reduced form, lactic acid (Grade L-1), and antimycin A (type III) were obtained from Sigma Chemical Co., St. Louis, MO. Bovine albumin, fraction V was obtained from Miles Laboratories, Elkhart, IN. All other chemicals were reagent grade. Portuguese Man-of-War nematocyst venom was prepared as described (6). The LD$_{50}$ of the venom (70 $\mu$g/kg body weight) was determined using female Swiss-Webster mice (21.4 ± 1.2 g) and noting the number of animals dead within two hr out of a group of eight animals per dose. The venom possessed 3.1 x 10$^4$ hemolytic units/mg and exhibited a hemolytic K$_{0.5}$ of 26 ng/ml (6).

Crude peritoneal mast cells were obtained from male Sprague-Dawley rats (weighting 500-600g), suspended in HEPES-buffered Locke's solution without heparin (LS-H), centrifuged for 5 min at 5°C and resuspended in LS-H (7). Mast cells were purified by sedimentation through 35% (w/v) bovine albumin dissolved in LS-H (8). The bottommost two ml of the cell suspension were resuspended in 5 ml LS-H and centrifuged at 50 x g for 5 min at 5°C. Cells from several animals were pooled in LS-H and enumerated using a hemocytometer. In a typical experiment, duplicate samples of cell suspensions (2.5 - 3.0 x 10$^4$ cells/ml) were equilibrated at 30°C for 10 min and then incubated with venom or compound 48/80 for 5 min. Mast cells depleted of ATP or of calcium were obtained by incubating in glucose- or
calcium-free LS-H plus antimycin A (5 mM), or EGTA (2 mM), respectively, and incubated for 10 min or 3 hr, respectively, before being treated with venom or 48/80. After treatment the samples were centrifuged at 7000 x g for 60 sec, using a Fisher centrifuge (Model 59). The supernatants were decanted and the cellular pellets suspended in LS-H. Both fractions were deproteinized with perchloric acid (0.4 N), centrifuged and then stored frozen until assayed for histamine.

Histamine was determined Fluorimetrically (9) with citric acid (2.0 M) substituted for HCl (10). Histamine release was calculated as the percent of the total histamine available in each cellular sample. Values for histamine release were corrected for spontaneous release, which was usually less than 5%. Lactate dehydrogenase (E.C. 1.1.1.27) released from mast cells was determined fluorimetrically (11) using a Zeiss PMQ III spectrophotometer equipped with a ZFM4 fluorescence attachment (FL39 barrier filter) and a XB0150 Xenon lamp. Lactate dehydrogenase released from cells was assessed in relation to that released by treating mast cells with 0.1% Triton X-100 (12).

RESULTS

Dose dependent histamine release.

Physalia venom causes the release of histamine from isolated mast cells in a dose-dependent manner (Fig. 1). The shape of the dose-response curve is hyperbolic with no detectable threshold. Maximum histamine release appears to approach 100% both in the presence and absence of calcium, although in the presence of calcium histamine release appears to be slightly inhibited. The concentration of venom which causes the release of 50% of the histamine ($K_{0.5}$) is 5.0 and 6.0 µg/ml in the
Figure 1. Dose-response of *Physalia* venom-induced histamine release with (o) and without (•) calcium. Data are means of duplicate samples with vertical lines showing range.
absence and in the presence of calcium, respectively.

**Calcium requirement for histamine release.**

Neither extracellular nor intracellular calcium (pre-incubation with EGTA in the absence of calcium) is required for venom-induced histamine release (Table 1). On the other hand, optimum histamine release induced by the secretagogue, compound 48/80, requires both extra- and intracellular calcium.

**Effect of temperature.**

Mast cells isolated and stored at either 0°C or room temperature were equilibrated and treated at different temperatures with compound 48/80 or venom and then incubated for 10 and 30 min, respectively (Fig. 2). Compound 48/80-induced histamine release is sharply optimized at 37°C with no significant histamine release occurring at either 0°C or 45°C. On the other hand, venom-induced histamine release is broadly optimized over the range of 10°C to 30°C with significant release at 0°C. Mast cells stored at 0°C, when treated with venom, release more histamine at all temperatures, except 0°C, than room temperature-stored cells. In contrast, cells treated with 48/80 yield identical temperature profiles regardless of storage temperature. At all incubation temperatures, except 0°C, venom-induced histamine release is completed by 30 min (Fig. 2, insert).

**Cytolytic effect.**

If histamine is released by a cytolytic process then there should be a concomitant or prior release of cytoplasmic constituents as well (13). Compound 48/80 causes release of histamine but not of the cytoplasmic marker, lactate dehydrogenase (Fig. 3). In contrast, the venom causes the release of both histamine and lactate dehydrogenase. Pre-
Table I. Influence of intra- and extracellular calcium on histamine release induced by Physalia venom or compound 48/80.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Histamine Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Venom</td>
</tr>
<tr>
<td>2 mM calcium</td>
<td>79.3</td>
</tr>
<tr>
<td>No calcium</td>
<td>87.7</td>
</tr>
<tr>
<td>2 mM EGTA, no calcium</td>
<td>76.6</td>
</tr>
</tbody>
</table>

Mast cells were preincubated for 3 hr with and without added calcium and with EGTA in the absence of calcium, and then treated with venom (11.3 μg/ml) or 48/80 (1.7 μg/ml). The data are means of duplicate samples from one representative experiment.
Figure 2. Influence of temperature on histamine-release induced by 
Physalia venom or compound 48/80. Mast cells isolated and 
stored at 0°C (open symbols) or at room temperature (closed 
symbols) were equilibrated and treated at the specified 
temperatures with venom (11.3 μg/ml; continuous lines or 48/80 
(1.5 μg/ml; broken lines). Vertical lines show range of 
duplicate samples. Insert: mast cells isolated and stored at 
room temperature were equilibrated and treated with venom for 
10, 20 and 30 min. The data are means of duplicate samples.
Figure 3. Histamine and lactate dehydrogenase release. Cells were treated with 48/80 (bars; mean ± S.E. of 4 samples) or venom (symbols), without (circles; mean and range of duplicate samples) and with (squares; mean ± S.E. of 8 samples) antimycin A. Histamine release (filled symbols; open bar) and lactate dehydrogenase release (open symbols; hatched bar) were assessed. With antimycin A present 48/80 induced the release of $2.2 \pm 0.8\%$ histamine and $0\%$ lactate dehydrogenase.
treating mast cells with antimycin A greatly increases the release of lactate dehydrogenase induced by the venom, but not of histamine. Antimycin A, however, totally inhibits the release of histamine induced by compound 48/80.

DISCUSSION

Since the classical work of Portier and Richet using tentacle extracts from Physalia and Actinia (3), our knowledge of the cellular and molecular basis of antigen-triggered anaphylaxis has grown considerably (14). Yet, the mechanism of cnidarian toxin-induced anaphylaxis has been left largely unstudied.

The issue of whether histamine release is caused by components of the nematocyst venom or by pharmacologically active substance(s) of extranematocyst origin is important in attempting to relate experimental findings to some of the symptoms manifested in sting victims and in suggesting rational modes of therapy. Since even cnidarian tissues normally devoid of nematocysts are known to possess potent, pharmacologically active substances (15), care should be taken to avoid attributing an observed pharmacological effect to the nematocyst toxins unless the substances are obtained directly from isolated nematocysts free of adhering debris. We have limited our study to a highly toxic mixture of soluble proteins from purified nematocysts isolated from the stinging tentacles of the Portuguese Man-of-War. We have operationally termed these soluble contents of the nematocysts, the nematocyst "venom".

The dose-response curve of Physalia venom-induced histamine release is hyperbolic in shape (Fig. 1) suggesting saturation binding to sites on the mast cell surface. The lack of a detectable threshold at low venom
concentrations implies that no critical number of venom molecules on the mast cell membrane are needed to cause histamine release and suggests single-hit kinetics (16). Histamine release approaches 100% at high doses of venom, well above the extent released by secretagogues (13).

In contrast to all known secretagogues (17), *Physalia* venom-induced histamine release is not decreased by the removal of either intra- or extracellular calcium (Table 1). In the presence of calcium venom-induced release is slightly diminished in a manner reminiscent of competitive inhibition (Fig. 1).

The action of the secretagogues is very temperature dependent (18) and optimal at 37°C with no activity at 0°C and 45°C (Fig. 2). This is also true of the *Cyanea* extract (5). *Physalia* venom, however, has a very broad temperature optimum from 10°C to 30°C, with decreased activity at 37°C. In addition, the venom is fairly active at 0°C, and the time-course at 0°C suggests that if given enough time the amount of histamine released would approach that of higher temperatures (Fig. 2, insert).

The fact that the cytoplasmic marker, lactate dehydrogenase, is released along with histamine in a dose-dependent manner from mast cells treated with *Physalia* venom but not with 48/80 (Fig. 3; ref. 12) strongly suggests that venom-induced release is cytolytic. Enzyme release is more sensitive to the venom than is histamine release, with half maximum release ($K_{0.5}$) occurring at 2.1 and 6.1 μg/ml for the enzyme and for histamine, respectively (Fig. 3). The fact that lactate dehydrogenase release is more sensitive to *Physalia* venom than is histamine release could mean either (i) that the histamine containing granules are not released from the cells until higher doses of venom produce larger sized
lesions in the plasma membrane, or else (ii) that the membranes of the secretory granules are more resistant to the cytolytic action of the venom.

Depletion of cellular ATP does not cause total inhibition of venom-induced histamine release (Fig. 3) as it does true secretagogues (19) and with Cyanea extract (5). ATP depletion, however, increases the sensitivity of venom-induced lactate dehydrogenase release tenfold ($K_{0.5} = 0.2 \mu g/ml$). Presumably, the plasma membranes of energy-depleted cells are more susceptible to lesion formation by the venom than are the membranes of energy-supplied cells.

In terms of the temperature profile and the requirement for cellular energy the action of the alcoholic extract from Cyanea tentacles on isolated mast cells (5) is quite different from that of the Physalia nematocyst venom. While this difference may be due to differences between species, it may also be due to the active component in the Cyanea extract being of extra-nematocyst origin.

In summary, we find that the venom from the Portuguese Man-of-War can cause the release of histamine from isolated rat peritoneal mast cells and that it does so by a cytolytic, rather than secretory, mechanism. Our conclusion is supported by our findings that release is neither dependent upon a cellular source of energy nor calcium. In addition, histamine release is fairly temperature insensitive, with the temperature optimum occurring below 37°C. Our conclusion, however, is based primarily on our observation that histamine release is accompanied by the release of lactate dehydrogenase; a widely held criterion for cytolysis of mast cells (12,13,17). The fact that more than 80% of the histamine
content of the mast cell is released by the venom also implicates a cytolytic process (13). Indeed the nematocyst venom from Physalia is known to possess a toxic and a extremely potent hemolytic protein, termed physalitoxin (6). Whether physalitoxin is responsible for the cytolytic release of histamine observed in the present study or for the anaphylactic-like symptoms shown by victims of Physalia stings remains to be determined. In the future we plan to identify the factor in Physalia venom which causes histamine release from mast cells and determine if it is identifiable in alcoholic extracts of whole tentacles.
Nematocyst venom from Portuguese Man-of-War (Physalia sp.) tentacles causes isolated rat peritoneal mast cells to release histamine. Extent of histamine release is dose-dependent ($K_{0.5} = 6.1 \mu g$ venom/ml) and attains 100% at high doses of venom. Release is independent of intra- and extracellular calcium levels and does not depend upon a cellular supply of ATP. The rate of histamine release is temperature-dependent and the extent of release is maximized broadly over the range of 10-30°C. The cytoplasmic marker lactate dehydrogenase, is released concomitantly with histamine but is more sensitive to the venom ($K_{0.5} = 2.1 \mu g/ml$). Antimycin A, while it does not significantly affect venom-induced histamine release, increases the sensitivity of lactate dehydrogenase release ($K_{0.5} = 0.2 \mu g/ml$). We conclude that Physalia nematocyst venom induces the release of histamine from mast cells by a cytolytic mechanism and that this action is antagonized by an intracellular, energy-requiring process.
REFERENCES


Appendix: Historical Review
Historical Review:

Discovery of Anaphylaxis

Richet (1) on an expedition in 1901 observed that the Portuguese Man-of-War (*Physalia physalis*) is capable of capturing prey much larger than itself and that divers stung by it suffered severe pain and fainting. Using saline, sea water, glycerol and alcohol extracts of *Physalia* tentacles he showed that experimental animals (pigeon, duck, guinea pig and dog) experienced immobility, sleepiness, dyspnea, insensitivity, convulsion, itching and death. The alcohol extract produced itching in the guinea pig. Generally, all four extracts produced the same symptoms but toxin extracted in saline produced the most potent effects, the dominating effects being insensitivity to stimuli and "invincible sleepiness".

Returning to France Richet collaborated with Portier (1) to continue the study. Since they were unable to obtain *Physalia* they used extracts from the sea anemone, *Actinia* (*Anemonia*) *sulcata*. From its macerated tentacles these investigators eventually were able to obtain three physiologically and chemically distinct toxins. Treatment of macerated tentacles with sodium fluoride and alcohol, water, and alcohol yielded the toxins congestine, hypotoxin and thalassine, respectively. When these were intravenously injected into dogs they produced vomiting and intestinal congestion, sedation, and severe pruritus with sneezing, respectively. Generally, the effects were the same as those observed using *Physalia* extracts and were most conspicuous in the dog.
Attempts were also made to immunize experimental animals with a glycerol extract of Actinia tentacles. Dogs were injected with heat-inactivated toxin. Several days later they received secondary injections of sublethal amounts of non-heated toxin and immediately experienced severe pruritus and insensitivity followed by death. Richet and Portier (1) called the phenomenon of becoming sensitized to the toxin, anaphylaxis. This classical description of anaphylaxis has been extended by numerous investigations and forms the foundation of our current knowledge of allergic atopy and anaphylaxis.

The Role of Histamine and Mast Cells in Anaphylaxis

**Histamine.** Dale and Laidlaw (2) showed that the effects produced by histamine in the guinea pig resembled those of anaphylaxis. Several years later Lewis (3) demonstrated that a histamine-like agent was liberated in the skin during local anaphylaxis. That same year Best and associates (4) established that histamine is present normally in the tissues of the body and suggested that it is released as a result of cellular injury. In the ensuing decade direct evidence was presented establishing that histamine is released during the anaphylactic reaction (5). Although histamine had been confirmed as a mediator of anaphylaxis the site and control of its release were still unknown.

**Mast cells.** Mast cells were first observed by von Reckingshausen (6) as granular cells in connective tissue. Ehrlich (7) was first to clearly describe and characterize these cells, and named them. They are widely distributed through the body, especially populous in connective tissues, lying along small blood vessels, and in many organs (e.g. liver,
heart, kidney). The mast cell exhibits great variation in form (ovoid, spherical, fusiform) and size (3.5 to 24 μm in diameter). Its cytoplasm contains an abundance of spheroidal basophilic granules which are often so numerous that they obscure other cytoplasmic structures (8).

Chemical studies of the mast cells were initiated in 1937 (9, 10), but it was not until the early 1950s that histamine was detected in them. In 1953, Riley and West (11) showed that there was a striking parallel between the histamine content and the numbers of the mast cells in various tissues. Keller and Schwarz-Speck (12), and Lagunoff (13) used isolated mast cells to provide the evidence that established that histamine is contained in the mast cells. Upon being exposed to a secretagogue mast cells are induced to release their histamine. Thus, mast cells mainly function as the production, storage and release sites for heparin and histamine. Heparin and histamine are complexed with zinc and are contained within the basophilic granules (14).

Cnidarian Venoms as Histamine Releasers

Many substances cause the direct release of histamine, that is, independently of hypersensitization mediated by specific antibody. Among them are the venoms and toxins of various organisms such as cnidarians, snakes and insects, which cause itching, reddening and local edema upon injection into the human skin (15). From the 1930s through the 1950s many reports appeared which attributed the effects of the cnidarian sting to the presence of histamine and/or serotonin-like agents in the tissue of cnidarians (16). This idea later fell into disfavor when
studies of isolated nematocysts failed to find vasoactive amines among the nematocyst toxins (17) but instead pointed to the toxins being proteinaceous (17,18,19).

In 1954, Jaques and Schachter (20) showed an alcohol extract of Actinia tentacles to be an effective histamine liberator. It caused histamine to be released from the perfused cat skin and raised the plasma histamine concentration following intravenous administration. Uvnäs (21) showed that a crude alcohol extract of jellyfish (Cyanea capillata) tentacles caused the release of histamine from isolated mast cells. Both these studies and those of Richet and Portier (1) used whole tissue, alcohol extracts, and, thus, it could not be determined if the direct pruritic and histamine releasing effects were due to constituents of the nematocysts or some non-nematocyst constituents of the tissues.

Besides these studies, the other work studying the direct effect of cnidarian venoms on mast cells is the recent work by Cormier (22), reported simultaneously with our study (see footnote on page 1). She reported that Physalia venom causes degranulation of isolated mast cells, accompanied by the release of histamine. She showed that venom-induced degranulation occurred more slowly than is reported for secretagogues and suggested that this indicates a different triggering mechanism of exocytosis. This disagrees with our findings and basic conclusion. We found that venom-induced histamine release is by a cytolytic, rather than an exocytotic, process. This is supported primarily by our observation that lactate dehydrogenase is released concomitant or prior to histamine release.
In our work we were also able to achieve 100% histamine release as opposed to a maximum of 40% total release reported by Cormier (22). This may be due to her working with a lower quality venom. When preparing the venom she used a pH which is not optimum for the venom stability. Another point of disagreement is her practice of adding cold buffer to stop the effect(s) of the venom on the mast cells. But as we have shown the venom is active even at low temperatures. In contrast to secretagogues, the venom is fairly active at 0°C and, given enough time, total histamine release will approach that of higher temperatures. Though we disagree on the mechanism of release we agree that a protein or proteins in the venom are responsible for the direct histamine-releasing effect of the venom.

The purpose of our present study was to investigate the direct histamine-releasing effect of Physalia venom on isolated mast cells and to determine the general mechanism involved. This study extended the earlier studies of Richet and Portier (1), Jaques and Schachter (20), and Uvnäs (21) by using venom obtained directly from isolated nematocysts and by describing the general mechanism by which the nematocyst venom caused histamine release from isolated rat peritoneal mast cells.
References


