Neutrophil Defensins Induce Histamine Secretion from Mast Cells: Mechanisms of Action

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Defensins are endogenous antimicrobial peptides stored in neutrophil granules. Here we report that a panel of defensins from human, rat, guinea pig, and rabbit neutrophils all have histamine-releasing activity, degranulating rat peritoneal mast cells with EC_{50} ranging from 70 to 2500 nM, and between 45 and 60% of the total histamine released. The EC_{50} for defensin-induced histamine secretion correlates with their net basic charge at neutral pH. There is no correlation between histamine release and antimicrobial potency. Degranulation induced by defensins has characteristics similar to those of activation by substance P. The maximum percent histamine release is achieved in <10 s, and it can be markedly inhibited by pertussis toxin (100 ng/ml) and by pretreatment of mast cells with neuraminidase. These properties differ from those for degranulation induced by IgE-dependent Ag stimulation and by the calcium ionophore A23187. GTPase activity, a measure of G protein activation, was induced in a membrane fraction from mast cells following treatment with defensin. Thus, neutrophil defensins are potent mast cell secretagogues that act in a manner similar to substance P and 48/80, through a rapid G protein-dependent response that is mechanistically distinct from Ag/IgE-dependent mast cell activation. Defensins may provide important pathways for communication between neutrophils and mast cells in defenses against microbial agents and in acute inflammatory responses. The Journal of Immunology, 1999, 163: 947–953.
the actions of ACTH on the adrenal glands (32, 33). They are also chemotactic for human monocytes (34), and at least two guinea pig defensins can induce histamine release from rat peritoneal mast cells (PMC) (35). Given this single report about the ability of defensins to induce histamine secretion and the possibility that neutrophil-derived HRF activity involves defensins (27, 28), we characterized the ability of several defensins to induce histamine secretion and studied their mode of action.

Materials and Methods

Animals

Outbred male Sprague Dawley rats (375–450 g) purchased from Charles River Canada (St. Constant, Canada) were used. They were maintained in an isolated room in filter-top cages to minimize unwanted infections. The animals were given food and water ad libitum and were maintained on a 12-h light (0700 h), 12-h dark (1900 h) cycle. No rats were used for experimentation until a minimum of 1 wk following arrival in our facilities, so as to reduce the effects of stress associated with transport, handling, and new environmental conditions. All experimental procedures were approved by the University animal care committee and were performed in accordance with the guidelines of the Canadian Council on Animal Care.

Either normal rats or rats infected 35–100 days previously with 3000 third stage larvae of the nematode, *Nippostrongylus brasiliensis* (to induce mast cell hyperplasia and IgE-dependent sensitization) were used to isolate PMC for study (8).

Mast cell isolation and enrichment

Normal rats or rats previously infected with *N. brasiliensis* were anesthetized, and 15 ml of cold Tyrode’s solution containing 12 mM HEPES and 1% BSA, pH 7.3 (HTB), were injected into the peritoneal cavity. The peritoneal exudate cells were layered on a 30%/80% discontinuous gradient of Percoll (Pharmacia, Dorval, Canada) for 20 min at 37°C (42) or with type V neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, MO) for 1 h at 37°C (43). Cells were washed twice with 1 ml of HTB and were resuspended in HTB for subsequent studies of their responses to secretagogues.

Histamine secretion and its modulation by pertussis toxin and neuraminidase

For studies of histamine secretion, 50,000 highly enriched PMC (≥98%) and the secretagogues to be used were preincubated separately at 37°C for 5 min, and then the secretagogues were added to the cells and incubated at 37°C for 10 min. The reaction was terminated by the addition of 1 ml of cold HTB and was centrifuged at 4°C to separate the supernatant and cell pellet. After the pellets were brought to 1.5 ml using HTB, the samples were boiled for 10 min to release cell-associated histamine and destroy histaminase activity. After TCA precipitation of proteins, histamine levels were measured in both supernatant and pellet fractions by fluorometric assay (36) using a model LS-3B Perkin-Elmer fluorescence spectrophotometer (Norwalk, CT). Histamine release was expressed as a percentage of the total cellular histamine content as calculated by the formula: (histamine in supernatant/histamine in supernatant and pellet) × 100. Spontaneous release of histamine was normally <2% of the total and was subtracted from the percent release to give the specific secretion shown in the text, tables, and figures. From dose-response curves of various secretagogues, the concentrations that specifically release 50% of the cellular histamine were calculated (EC50).

The secretagogues used in the studies included an extract of *N. brasiliensis* Ags (8), the neuromedepptide substance P (10–8 M), the ionophore A23187 (5 × 10−6 M), and a panel of defensins from humans, rabbits, and guinea pigs. Defensins were isolated from bone marrow for the rabbit (37) and guinea pig (38) peptides and from leukocytes obtained from the peritoneal exudate of patients with peritonitis for the human peptides (39, 40). The peptides were purified by reverse phase and size exclusion chromatography as previously described (37–40). The identity, purity, and quantity of each peptide were determined by amino acid analysis. Purified peptides were stored lyophilized at −20°C until use. The nomenclature and structure of the defensins used in this manuscript are summarized in Table I. The guinea pig peptide GPCSIII is more closely related to the protegrin family (41) of cysteine-rich cationic neutrophil peptides than to the classic defensins and exists as an antiparallel homodimer (38).

To compare the time course of histamine secretion induced by various secretagogues, the secretory reaction was abruptly terminated at various times following mixing the secretagogue and cells by adding 1 ml of ice-cold HTB, placing the tubes immediately on ice, and then separating into supernatant and pellet fractions by centrifugation at 180 × g for 5 min at 4°C. In several experiments, highly enriched mast cells were pretreated with pertussis toxin (List Biochemical Laboratory, Campbell, CA) for 2 h at 37°C (42) or with type V neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, MO) for 1 h at 37°C (43). Cells were washed twice with 1 ml of HTB and were resuspended in HTB for subsequent studies of their responses to secretagogues.

Isolation of mast cell membrane fraction

A mast cell membrane fraction was isolated as described previously (44). Briefly, purified PMC (30 × 10^6) were resuspended in homogenization buffer (HEPES buffer with 2 mM MgCl2, 1 mM ATP, 100 μg/ml PMSF, and a protease inhibitor mixture consisting of 5 μg/ml each of leupeptin, aprotonin, and tosyl-arginine methyl ester (TAME)). Cells were ruptured by repeated passage (5–10 times) through a 12-μm clearance in a ball-bearing homogenizer (EMBL, Heidelberg, Germany), with resulting organelles removed by centrifugation at 400 × g for 10 min. The clear supernatant was then centrifuged at 100,000 × g for 1 h at 4°C. The membrane pellet was resuspended in a small volume of 10 mM triethanolamine/HCl buffer and was stored at −70°C until use.

GTase assay

GTase activity was assayed by the hydrolysis of phosphate, from [γ-32P]GTP as previously described (45). Briefly, a reaction mixture (100 μL) containing 50 mM triethanolamine/HCl (pH 7.5), 2 mM MgCl2, 1 mM DTT, 0.1 mM EGTA, 2 mg/ml BSA, 0.8 mM adenosine 5’-(β,y-imino)-triphosphate, 0.1 mM ATP, 0.4 mg/ml creatine kinase, 5 mM creatine phosphate, and 5 mM [γ-32P]GTP was prewarmed (5 min, 30°C). Reactions were started by addition of membrane fraction (1.0–2.0 μg of protein) to the prewarmed incubation mixture containing substance P or NP3a and allowed to proceed for 25 min at 30°C. The reaction was stopped by addition of 700 μL of 10 mM sodium phosphate (pH 2.0) containing 5% (w/v) activated charcoal. The tubes were chilled for 30 min on ice, then centrifuged at 13,000 × g for 10 min. A 500-μL aliquot was removed, and GTase activity was evaluated by determination of Cherenkov radiation.

### Table I. The nomenclature and primary structures of defensins tested for mast cell degranulating activity

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>NP3α/CSI</th>
<th>GICACRRRF-CPSERFGYCRVNGARYVCGR CRR</th>
<th>P</th>
<th>C-G—G—CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP3β/CSI</td>
<td>GRVCRKLQCSSYRRIDCKIRGVPPFFCPR</td>
<td>VVCA CRRAL-CLPERRAGFCRGRHP LCCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP1/CSI</td>
<td>ACYRPA-CA GERRYGTCIQYGRLWAFCC</td>
<td>VCS CRLVF- CRKTELVRGNC LGVS FYCTR V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>ACECITRTRCFPPYRLGTCIFQRNYTFCC</td>
<td>RRPRFCRCLHR CRC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The secretagogues used in the studies included an extract of *N. brasiliensis* Ags (8), the neuromedepptide substance P (10–8 M), the ionophore A23187 (5 × 10−6 M), and a panel of defensins from humans, rabbits, and guinea pigs. Defensins were isolated from bone marrow for the rabbit (37) and guinea pig (38) peptides and from leukocytes obtained from the peritoneal exudate of patients with peritonitis for the human peptides (39, 40). The peptides were purified by reverse phase and size exclusion chromatography as previously described (37–40). The identity, purity, and quantity of each peptide were determined by amino acid analysis. Purified peptides were stored lyophilized at −20°C until use. The nomenclature and structure of the defensins used in this manuscript are summarized in Table I. The guinea pig peptide GPCSIII is more closely related to the protegrin family (41) of cysteine-rich cationic neutrophil peptides than to the classic defensins and exists as an antiparallel homodimer (38).
Statistical analyses

Data are given as the mean ± SEM. Differences among treatment groups were examined for significance by two-way ANOVA. Individual differences were assessed for statistical significance (p < 0.05) by paired or unpaired Student’s t test.

Results

Dose response and EC_{50} for defensins

The abilities of various concentrations of rabbit, guinea pig, and human defensins to induce histamine secretion from purified rat PMC were studied (Fig. 1). The rabbit defensins NP1, NP3a, and NP3b induced histamine secretion at low nanomolar concentrations and induced a maximum of 50–60% specific histamine release. The EC_{50} for these rabbit peptides varied from 70–620 nM (Table II). A similar pattern of histamine secretion occurred with guinea pig defensins, GPNP1 and GPCSIII (Fig. 1B and Table II). The human defensins HNP1 and HNP4, were less potent in inducing histamine secretion from rat mast cells. At 2.5 μM the maximum histamine secretion was 40–55% with EC_{50} for HNP1 and for HNP4 of 2.9 and 2.2 μM, respectively (Fig. 1C and Table II).

Although the amounts of rat defensins available for study were limited, in a single experiment R2 (5 nM), R4 (0.5 nM), and R5 (0.5 nM) induced histamine secretion (15.2, 51.7, and 45.8%, respectively); R3 induced no histamine secretion.

The EC_{50} of the defensins studied generally correlated with the nominal net charge of the peptides at pH 7.4 as calculated from their known sequences (Table II). These values correlate with their relative charge as determined by acid-urea gel electrophoresis. Rabbit NP3a was the most potent inducer of specific histamine release (EC_{50} = 70 nM; Table II). Thus, for further studies of the mechanisms involved in histamine secretion with defensins we used NP3a as a representative peptide. For comparisons, the characteristics of histamine secretion induced by NP3a under different experimental conditions (time-course analysis and effects of pertussis toxin and neuraminidase) were compared with those of substance P, Ag, and the calcium ionophore A23187. Interestingly, NP3a did not induce histamine secretion from mucosal mast cells isolated (8) from rat small intestinal mucosa (results not shown).

Time course analysis of histamine secretion

Given that differences in the time course of histamine secretion occur with various secretagogues (polycations have a rapid time course, ≤10 s) and relate to the activation pathways involved, we studied the time course of histamine secretion induced by NP3a and other secretagogues. NP3a and the polycation substance P exhibited a rapid time course (<10 s required for maximum percent histamine release), whereas the time courses of Ag- and ionophore (A23187)-induced histamine secretion were delayed and reached a maximum after 20 or at least 60 s, respectively (Fig. 2).

Effects of pretreatment with pertussis toxin on histamine secretion

Histamine secretion induced by polycations such as 48/80 and substance P is highly sensitive to pertussis toxin, whereas Ag- and ionophore-induced histamine release is not (10, 13, 46–48). Therefore, we investigated the effects of pertussis toxin on NP3a-induced histamine secretion (Fig. 3). Purified PMC were preincubated for 2 h with various concentrations (1–100 ng/ml) of pertussis toxin, washed, and then stimulated with various secretagogues for 10 min. Pertussis toxin (100 ng/ml) almost completely inhibited histamine secretion induced by substance P (94.5 ± 3.8% inhibition) and NP3a (96.3 ± 2.3% inhibition), whereas it was without effect on A23187-induced histamine secretion (data not shown) and only partially inhibited (48 ± 5.5%) Ag-induced histamine secretion. Inhibition was evident with as little as 10 ng/ml pertussis toxin during a 2-h pretreatment.

Effects of pretreatment with neuraminidase on histamine secretion

Histamine secretion induced by polycations, but not by A23187, is also highly sensitive to inhibition by the removal of sialic acid residues from the cell surface using neuraminidase (10). Thus, to characterize the sensitivity of NP3a-induced histamine secretion to

FIGURE 1. Specific histamine secretion induced by 10-min exposure of purified rat peritoneal mast cells to defensins from: A, rabbit (NP1, NP3a, and NP3b); B, guinea pig (GPCS1, GPCSIII); and human (HNP1 and HNP4). Values are the mean ± SE (n = 4 separate experiments for each defensin). The percent histamine secretion for each of the rabbit peptides (A) is significantly different from the others at 50, 140, and 420 nM; for the guinea pig peptides (B) at 30, 90, and 280 nM; and for the human peptides (C) at 280, 830, and 2500 nM (p ≤ 0.05).
neuraminidase, we pretreated purified PMC with various concentrations (0.01–0.1 U/ml) of neuraminidase for 1 h, washed them, and then challenged them with various secretagogues (Fig. 4). NP3a- and substance P-induced histamine secretion was highly sensitive to the removal of sialic acid residues (80.0 ± 2.4% and 72.7 ± 8.0% inhibition, respectively). By contrast, histamine secretion induced by Ag stimulation was partially (33.8 ± 5.2%) inhibited by neuraminidase treatment, and there was no effect of neuraminidase on histamine secretion induced by A23187 (data not shown).

Enhancement of GTPase activity by NP3a

Because the activation of histamine secretion from mast cells by NP3a appeared to be similar to that of substance P and presumably other polycationic peptides, we studied whether NP3a could induce GTPase activity, a measure of activation of G proteins, in a membrane fraction of PMC. An isolated membrane fraction from purified PMC induced a significant (p < 0.05) GTPase response in the presence of 140 nM NP3a (7.7 ± 1.6 pmol/min/μg), consistent with or greater than the positive response to 10⁻⁵ M substance P (7.3 ± 1.7 pmol/min/mg; Fig. 5). This was reproducible in multiple studies with a single membrane preparation as well as with independent membrane preparations.

Discussion

Several cationic peptides, including venom peptides such as mast cell-degranulating peptide (MCDP), and neuroendocrine peptides such as substance P, vasoactive intestinal polypeptide, and somatostatin promote IgE-independent histamine release from mast cells. Rat intestinal mucosal mast cells often contact subepithelial substance P neurons (49), suggesting a physiological role for substance P-induced mast cell degranulation. This observation received strong support when Janiszewski et al. (9), showed that concentrations as low as 5 pM substance P primed mast cells for subsequent activation by substance P and other secretagogues. However, peptide-induced histamine release also may be an unwanted side effect in the development of pharmacologically useful peptide agents; for example, many luteinizing hormone-releasing hormone antagonists degranulate mast cells (50). Neutrophils elaborate low molecular mass (≤5 kDa) HRF whose identity is not known but which appears by biological and chromatographic criteria to differ from other biochemically characterized histamine-releasing polypeptides. Neutrophil-derived guinea pig defensins activate histamine release from mast cells (35). We therefore examined the histamine-releasing properties and mechanisms of action of several defensins from different species as candidate neutrophil-derived HRF.

The ability to degranulate mast cells is common to all defensins tested, with EC₅₀ ranging from 70 nM to 2.9 μM. Defensins resemble wasp MCDP in terms of their cysteine-rich character and their potencies on rat PMC: 20 nM for MCDP (51, 52) and 70 nM for NP3a. On a molar basis the EC₅₀ for the highly cationic defensins (net positive charge at pH 7.4 above 6) are lower or in the same range as those for anaphylatoxins C5a and C3a (53) on dermal mast cells and are orders of magnitude lower than those of

### Table II. Maximum histamine secretion, EC₅₀ and nominal net charge at pH 7.4 of defensins tested for mast cell degranulating activity

<table>
<thead>
<tr>
<th>Species</th>
<th>Peptides</th>
<th>Maximum Histamine Release (%)</th>
<th>Conc. (nM)</th>
<th>EC₅₀ (nM)</th>
<th>Net Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>GPNPI</td>
<td>67.4 ± 1.7</td>
<td>830</td>
<td>100</td>
<td>+7</td>
</tr>
<tr>
<td></td>
<td>GPCSIII</td>
<td>68.8 ± 1.7</td>
<td>2500</td>
<td>300</td>
<td>+10</td>
</tr>
<tr>
<td>Human</td>
<td>HP-1</td>
<td>43.1 ± 3.2</td>
<td>2500</td>
<td>2870</td>
<td>+3</td>
</tr>
<tr>
<td></td>
<td>HP-4</td>
<td>55.4 ± 3.2</td>
<td>2500</td>
<td>2160</td>
<td>+4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>NP3a</td>
<td>65.2 ± 1.8</td>
<td>420</td>
<td>70</td>
<td>+8</td>
</tr>
<tr>
<td></td>
<td>NP3b</td>
<td>55.0 ± 1.5</td>
<td>1260</td>
<td>240</td>
<td>+8</td>
</tr>
<tr>
<td></td>
<td>NP1</td>
<td>53.6 ± 3.1</td>
<td>1260</td>
<td>620</td>
<td>+9</td>
</tr>
</tbody>
</table>

FIGURE 3. Effects of pretreatment with pertussis toxin (2 h, 37°C) on the inhibition of specific histamine secretion stimulated by substance P (10⁻⁵ M), NP3a (140 nM), A23187 (5 × 10⁻⁶ M), or N. brasiliensis (five worm equivalents) Ag (mean ± SE; n = 3–6). NP3a and substance P are significantly different from Ag and A23187 at all doses of pertussis toxin (*, p ≤0.05).

FIGURE 2. Time-course analysis of specific histamine secretion induced by substance P (10⁻⁵ M), NP3a (140 nM), A23187 (5 × 10⁻⁶ M), or N. brasiliensis (five worm equivalents) Ag (mean ± SE; n = 3–6 experiments for each secretagogue). NP3a and substance P are significantly different from Ag and A23187 at 5 and 10 s (*, p ≤0.05).
nonlyncysteinyl neuroendocrine peptides (8) such as somatostatin (EC$_{50}$, ~2.0 μM), vasoactive intestinal polypeptide (EC$_{50}$, ~6.0 μM), and substance P (EC$_{50}$, ~40.0 μM) on rat PMC. The range of defensin EC$_{50}$ as mast cell secretagogues shows a strong correlation with the net charge of the peptide at pH 7.4; the more basic defensins are more potent mast cell-degranulating agents. The potency of mast cell degranulation also tends to follow the rank order of these peptides as inhibitors of the ACTH receptor (corticostatic activity), suggesting similarities in the underlying mechanisms of these two responses. HP-1 and HP-4, which have the weakest histamine-releasing activity are antimicrobial (54, 55), demonstrating that the structure-activity correlates that govern histamine secretion differ from those that determine microbicidal action.

In common with other cationic peptides, defensins degranulate mast cells through a pertussis toxin-inhibitable mechanism, implying an action on G proteins. The time course of histamine release by defensins is identical with that of substance P, and both peptides are inhibited by neuraminidase treatment of mast cells. Moreover, as with substance P, NP3a induces GPase activity. Thus, the mechanism of defensin-induced mast cell degranulation is similar to that of other polycationic peptides. This is also consistent with the lack of effect of NP3a on histamine secretion from intestinal mucosal mast cells, as this mast cell subset is unresponsive to other polycations. This unresponsiveness may protect these mucosal mast cells from spurious activation by defensin-like cryptdins thought to be constitutively released from Paneth cells in the intestinal crypts (56). Alternately, cryptdins may activate mucosal mast cells, whereas neutrophil-derived defensins do not. Peptide-induced histamine release is thought to involve receptor-independent activation of G proteins (13). The details of how such peptides activate G proteins remain unclear, but involve direct interaction of the peptide with the G protein. Charge and membrane penetration are important; however, HP-1, which is membrane penetrating (57), only weakly activates mast cell histamine secretion. In studies on synthetic magainin analogues (amphipathic helical antimicrobial peptides from frog skin) Cross et al. (58) were able to uncouple strong histamine-releasing activity from peptide-induced membrane perturbations. The amino acid configuration may exert an important influence on peptide-induced mast cell degranulation, since L- to D-arginine substitutions in substance P analogues, which conserved overall charge, showed reduced mast cell activation (58).

There is growing evidence of important communication between mast cells and neutrophils in inflammatory reactions. Neutrophil recruitment to inflammatory sites is mast cell dependent (59), involves their production of TNF-α (60), and has important antimicrobial activities (61, 62). In addition, mast cell-derived tryptase has a pronounced effect on neutrophil recruitment (63, 64). In turn, neutrophil-derived factors have long been implicated in histamine release at sites of inflammation, and defensin-induced histamine release may contribute to this process. During infection, plasma defensin concentrations may greatly exceed the levels that induce degranulation of mast cells in vitro. In sepsis, cumulative concentrations in plasma of the human defensins HNP1–3 rise from 40 ng/ml in healthy individuals to as high as 170 ng/ml (65, 66). Highly elevated levels of HNP1–3 occur in sputum from cystic fibrosis patients (67) and in pleural effusions from patients with empyema (68). Solid tissue infiltrated with neutrophils may have total HNP1–3 levels up to 25 nmol/g wet weight or roughly 90 μg (39). Defensins are prominent within the extracellular spaces of experimentally induced syphilitic lesions in rabbits (69). The plasma level of rabbit NP3a, the most active MCDP, increases from 8 ng/ml in uninfected animals to 234 ng/ml during experimental peritonitis (70). The release of defensins may arise both from the lysis of expended neutrophils and from cytokine-stimulated secretion. IL-8 (64, 68) or PMA (68) stimulates the release of HNP1–3 from human neutrophils, and at least in pleural effusions the local levels of IL-8 exceed the concentrations required to promote the secretion of neutrophil defensins.

Since defensins are released from neutrophils in response to IL-8 and can be proinflammatory (71), these peptides may also be released by mast cell-derived TNF-α and tryptase and accumulate at sites of inflammation. Thus, it appears that this bidirectional communication between mast cells and neutrophils is important in acute inflammatory responses. It may involve important innate responses that help direct the kinds of immune and inflammatory responses that evolve following injury and Ag exposure and clearly requires further study. Regardless of the physiological or pathological outcomes of defensin-induced histamine release and inflammatory cascades, the design of novel antibiotics based on defensin structures will have to take account of their propensity to degranulate mast cells.

**FIGURE 4.** Effects of pretreatment with neuraminidase (1 h, 37°C) on the inhibition of specific histamine secretion stimulated by substance P (10$^{-5}$ M), NP3a (140 nM), A23187 (5 × 10$^{-6}$ M), or N. brasiliensis (five worm equivalents) Ag (mean ± SE inhibition, n = 4–5; n = 2 for A23187). NP3a and substance P are significantly different from Ag and A23187 at neuraminidase concentrations of 0.025, 0.05, 0.075, and 0.1 U/ml (*, p ≤ 0.05).

**FIGURE 5.** Increase in hydrolysis of phosphate from [γ-32P]GTP (GPase activity) following treatment of membrane fraction isolated from mast cells with substance P (10$^{-5}$ M) or rabbit defensin, NP3a (140 nM), at 30°C (results of three experiments from two independent membrane preparations: *, p < 0.05).
Acknowledgments

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References


