Histamine Distribution in Human Basophil Secretory Granules Undergoing FMLP-Stimulated Secretion and Recovery

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We examined subcellular histamine localizations in purified human basophils that were stimulated to degranulate with FMLP using an ultrastructural enzyme-affinity technique. Basophils were collected at early (0, 20 seconds, 1 minute) and late (10 minutes to 6 hours) time points poststimulation and were prepared for routine ultrastructural and diamine oxidase-gold (DAO-gold) cytochemical analysis. Histamine was present in unaltered cytoplasmic secretory granules (30.77 gold particles per square micrometer; \( P < .001 \)) compared with background; specificity controls (histamine absorption, diamine oxidase digestion) abrogated granule labeling for histamine. Altered granules in stimulated cells were not significantly labeled for histamine, as compared with background (\( P = \) not significant); unaltered granules in the same cells contained more histamine than altered granules (\( P < .05 \)). During recovery times, spanning 10 minutes to 6 hours, granules again appeared to be electron-dense and contained histamine (33.49/p.m.\(^2\); \( P = \) not significant as compared with unaltered granules in 1-minute FMLP-stimulated cells, and \( P < .05 \) as compared with altered granules in 1-minute FMLP-stimulated samples). Other structures devoid of histamine in actively secreting cells included extruded granules and intragranular and extruded Charcot-Leyden crystals. Recovering basophils displayed morphologic evidence of material and membrane conservation, granule content condensation, and biosynthesis. Subcellular histamine-rich sites in actively recovering basophils included condensing granules and collections of cytoplasmic vesicles in three locations: beneath the plasma membrane, adjacent to granules, and in the Golgi region. These studies show that unaltered granules of actively releasing human basophils, as well as similar granules that are reconstituted after FMLP-stimulated degranulation, contain histamine, but that altered granules in stimulated cells undergoing degranulation are devoid of histamine. Reconstitution of histamine-rich granules is associated with DAO-gold-positive cytoplasmic vesicles, suggesting transport of histamine derived from either new synthesis, re-uptake of released histamine, or both, to reconstituted granules.

GRANULOCYTES were once considered to be the cell source of histamine in human peripheral blood.\(^1\) The subcellular location of histamine in human leukocytes was found by subcellular fractionation to be within granules, structures shown to differ from lysosomes by their lack of \( \beta \)-glucuronidase.\(^2\) Histamine release from washed leukocytes of allergic individuals was demonstrated after incubation with specific antigen.\(^3\) Since these initial studies,\(^1,3\) it has become clear that (1) basophils represent the single intravascular cellular source of peripheral blood histamine in humans and (2) a wide variety of secretagogues induce regulated histamine secretion from basophils. The ultrastructural anatomy and cellular biochemistry of these various release reactions have been recorded in detail.\(^4,7\) We have defined two general anatomic release reactions of human basophils (HBs): anaphylactic degranulation (AND), indicating massive and rapid extrusion of granules to the extracellular milieu, and piecemeal degranulation (PMD), indicating the release of focal and complete granule contents in the absence of granule-granule or granule-plasma membrane fusions.\(^4,5\) The anatomic result of the PMD process is retention of partially empty and empty granule containers in the cytoplasm of viable cells—a process that was initially described in HBs that migrated into contact sensitivity skin reactions, but one that has subsequently been documented in basophil and mast cells in multiple sites, species, and diseases.\(^8\) Ultrastructural studies of FMLP-stimulated degranulation and recovery of HBs have revealed the rapid onset of an overlapping continuum of the morphologies of PMD and AND: PMD occurred in seconds, and AND peaked by 1 minute. Early recovery changes (the onset of which was evident at 5 to 10 minutes) were extensive but incomplete by 6 hours (Dvorak et al\(^9\) and A.M.D., unpublished data, January 1995). In the study reported here, we used a new enzyme-affinity–gold ultrastructural method that is based on the affinity of diamine oxidase (histaminase) for histamine\(^10\) to examine the distribution of histamine in HBs expressing the morphologies of PMD and AND and compared these findings to the histamine distribution in HBs recovering from FMLP-induced secretion.

MATERIALS AND METHODS

Basophil purification. Buffy coat cells, obtained from normal donors undergoing hemapheresis, were partially purified by countercurrent centrifugal elutriation and then placed on Percoll density gradients (1.075 and 1.066 g/mL). Purified basophils were recovered from the interface between the two Percoll layers.\(^11\) Two final preparations contained 52% and 28% basophils. A total of 1,800 basophils were examined by electron microscopy.

Histamine release assay. Cells were suspended in PAGCM (PIPEs [piperazine-N,N′-bis-2-ethane sulfonic acid]-albumin-glucose supplemented with 1 mmol/L CaCl\(_2\) and 1 mmol/L MgCl\(_2\) ) and stimulated with 2 \( \mu \)mol/L or 5 \( \mu \)mol/L FMLP at 37°C for 10 or 30 minutes. The supernatant was removed and assayed for histamine release, as before.\(^6\) Spontaneous histamine release was measured in replicate unstimulated samples. FMLP-stimulated histamine release for the two preparations was as follows: 10-minute sample, 30% (14% spontaneous release); 30-minute sample, 39% (11.7% spontaneous release).

Electron microscopy. Aliquots of cells, either stimulated with

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(and recovered in the presence of) FMLP or not, yielded 36 individual samples that were prepared for electron microscopy by immersion fixation of suspended cells, as before,12 at time 0, 0.20 seconds, 1 minute, 10 minutes, 20 minutes, 30 minutes, 1 hour, 3 hours, and 6 hours. Cells from some samples were resuspended in Hanks' balanced salt solution containing 50 μL (0.5 mg) cationized ferritin (Miles Laboratories, Inc, Kankakee, IL), on a rotary shaker set at low speed for 30 minutes at room temperature. Further processing and embedding was as reported.12 Thin sections were stained with lead citrate and viewed in a Philips 400 electron microscope (Philips North America, Mahwah, NJ).

Diamine oxidase-gold affinity stain for histamine. Thin sections of Epon-embedded material were prepared to demonstrate histamine with the ultrastructural enzyme-affinity method, based on diamine oxidase-gold (DAO-G) binding. Specificity controls included prior digestion of samples with diamine oxidase or absorption of the DAO-G reagent by solid-phase histamine. We manually counted 376 gold particles, and a digitizer was used to measure 11.57 μm² of granule area and 9.30 μm² of Epon background. The density of gold particles per square micrometer was determined for 20 full (electron-dense) granules in 20 different cells, for 10 altered (electron-lucent) granules in 10 different cells, and for comparable areas of Epon background in each of 30 prints. Statistical differences were established by χ² analysis.

RESULTS

DAO-G affinity stain: An ultrastructural enzyme-affinity technique that specifically labels histamine in HB granules of unstimulated cells. HBs have large secretory granules that are membrane-bound and contain closely packed electron-dense particles. Concentric dense membranes and homogeneously dense Charcot-Leyden crystals (CLCs) are also irregularly present in this granule population.1 DAO-G bound to secretory granules that were filled with electron-dense particles, as evidenced by gold labeling of these structures in unstimulated cells (Fig 1A). The specificity of binding for histamine was demonstrated by abrogation of gold-labeling of granules when the specific DAO-G reagent was absorbed with solid-phase histamine before use in the electron microscopic staining procedure (Fig 1B), as well as by the absence of granule labeling in thin sections that were digested with DAO before staining with the DAO-G reagent (Fig 1C). Thus, specific substrate (histamine) and specific enzyme (DAO) controls removed the ability of DAO-G to label histamine in HB granules of unstimulated cells.

Histamine distribution in secretory granules of HBs stimulated by FMLP to release histamine. Extensive histamine release occurs between 20 seconds and 1 minute after stimulation of HBs with FMLP. These time intervals were, therefore, selected to investigate histamine distribution in secretory granules during this process. Secretory granules of FMLP-stimulated HBs showed several major changes. These included altered granule content, characterized by less dense particle packing (which resulted in partially empty and empty granule containers), and extrusion of individual granule particulate structures to the cell surface (Fig 2). Granules with altered contents often contained CLCs, and CLCs were also extruded to the cell surface after fusion of individual granule membrane to the plasma membrane (Fig 2). DAO-G staining of human basophils with such granule alterations showed that unaltered cytoplasmic granules were labeled for the presence of histamine (Fig 2A and B) and that extruded granules, attached to the cell surface, generally were devoid of histamine (Fig 2C and D). CLCs within unaltered (Fig 2E) and altered (Fig 2F) cytoplasmic granules and CLCs after extrusion to the cell surface (Fig 2G) were devoid of histamine. Altered granules showing focal or complete release of granule particles did not contain histamine, either in piecemeal areas of granule particle release (Fig 2H) or in granules largely devoid of their particles (Fig 2F).
Fig 2. HB granules from cells stimulated for 1 minute with FMLP and prepared with DAO-G to label histamine. Gold is bound to the unaltered, membrane-bound cytoplasmic granules, which are completely filled with matrix particles (A and B), but not to the membrane-free, extruded, extracellular granule matrix masses attached to the cell surface (arrowheads, C and D). Nuclear (N) label of polyamines is present in panel D. A cytoplasmic vesicle (arrow) is labeled in panel C. (E) The particle-filled portion of a granule surrounding a central CLC (c) contains gold label. The intragranular CLC (c) is not labeled. Cytoplasmic vesicles are also labeled (arrows). The gold particles are slightly larger and denser than the nearby individual cytoplasmic glycogen particles. The altered, membrane-bound cytoplasmic granule in panel F has a central CLC (c), and the surrounding matrix has released all electron-dense particles. This granule contains no label for histamine; the CLC also is not labeled. An extruded CLC (c), attached to the cell surface, is not labeled for histamine in panel G. A cytoplasmic granule in panel H shows a focal piecemeal loss of particles that does not label for histamine; the surrounding particulate matrix is labeled for histamine. OM: A, C, and F, × 82,000; B, D, and G, × 38,000; E, × 83,500; H, × 54,000.

We quantified these findings (Table I) and showed that releasing HBs contained a density (gold particles per square micrometer) of 30.77/μm² in their unaltered, particle-filled granules, and 4.08/μm² in altered, particle-poor granules (P < .05). Neither the extruded particulate granules nor the granule-derived CLCs contained gold label in their extracellular location. The background gold density was 3.88/μm², a value significantly different from unaltered, particle-filled cytoplasmic granules (P < .001), but essentially the same as in altered, particle-poor cytoplasmic granules (P = not significant [NS]). Thus, it is clear that histamine is released from intracytoplasmic altered granules and is absent from extracellular granules at early times, concomitant with the biochemical detection of histamine release in FMLP-stimulated HBs.

Histamine distribution in secretory granules of HBs recovering from FMLP-stimulated secretion. At various recovery time intervals spanning 10 minutes to 6 hours after FMLP stimulation HBs morphologically reconstitute granule contents and no longer contain large numbers of granules with altered contents (Fig 3). Some extruded granule particles (Fig 2) and CLCs do persist at the earlier recovery times. As in the degranulation phase, these extracellular structures

### Table 1. Density of DAO-G Labeling in Human Basophil Granules Indicating Histamine During Secretion and Recovery

<table>
<thead>
<tr>
<th>Phase</th>
<th>Dense Granules</th>
<th>Altered Granules</th>
<th>Background</th>
</tr>
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<tbody>
<tr>
<td>Secretion</td>
<td>30.77 ± 16.1 (10)</td>
<td>4.08 ± 2.97 (10)</td>
<td>3.88 ± 0.9 (20)</td>
</tr>
<tr>
<td>Recovery</td>
<td>33.49 ± 4.21 (10)</td>
<td>5.25 ± 1.24 (10)</td>
<td></td>
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</tbody>
</table>

Density is expressed as number of gold particles per square micrometer ± SE. Numbers in parentheses indicate number of granules or Epon backgrounds counted.

* P < .05 (compared with altered granules).
† P < .001 (compared with dense granules).
‡ P < .05 (compared with altered granules or background).
did not label for the presence of histamine. In the recovery phase, HBs showed large numbers of cytoplasmic vesicles, granule content increases to baseline levels, and morphologic evidence of internalization of membrane and materials from the plasma membrane location (Fig 3). Use of cationized ferritin in the postfixation period allowed the identification of granules and/or empty granule containers that were in continuity with the cell surface (Figs 3 and 4). A few remaining empty granules contained large CLCs but no granule particles, and they were not in continuity with the cell surface (Fig 4A and B). In HBs recovering from FMLP-stimulated secretion, cytoplasmic vesicles and unaltered granules were labeled with DAO-G (Figs 3, 4C, and 5), indicating the presence of histamine. Large empty granules, either in continuity with the cell surface (Fig 3) or not so connected (Fig 4A and B), were devoid of histamine. CLCs within these altered (Fig 4A and B) and unaltered (Fig 4C) granules also did not stain with DAO-G. Granule particles remaining on the cell surface, or in the process of re-internalization, contained very little histamine (Fig 4E). The numerous reconstituted, unaltered granules present in recovering HBs contained histamine (Figs 3, 4C and D, and 5).

Quantitation of the DAO-G labeling in unaltered dense granules of recovering HBs (Table 1) showed a density of 33.49/µm², a value significantly higher than that of histamine labeling in altered granules of actively secreting basophils (4.08/µm²; \( P < .05 \)) and identical to the unaltered dense granules in actively secreting cells (30.77/µm²; \( P = \text{NS} \)). Background density of labeling was 5.25/µm², a value significantly less than that of dense granule labeling in recovering basophils (\( P < .05 \)). Significant differences in background gold particles were not present when degranulation and recovery experiments were compared (\( P = \text{NS} \)).

The area of dense granules and altered granules that was used for the DAO-G analysis was also measured in these experiments. Dense granules, whether present in actively degranulating (0.30 µm) or recovering (0.42 µm) basophils, did not differ in size (\( P = \text{NS} \)). Also, neither dense-granule population differed significantly in size from the histamine-free, altered granules that were present in actively degranulating cells (\( P = \text{NS} \)).

**DISCUSSION**

We used a newly developed enzyme-affinity-gold technique to label histamine in ultrastructural samples of HBs undergoing an overlapping, FMLP-stimulated continuum of PMD to AND, followed by recovery from these secretory events. These new studies confirmed that the granule stores of histamine were in membrane-bound cytoplasmic granules, which were filled with electron-dense particles. The density of histamine labeling was virtually the same in the electron-dense particle-filled granules of cells stimulated for 1 minute with FMLP (ie, 30.77/µm²) as in the electron-dense particle-filled granules of basophils recovered 10 minutes to 6 hours after FMLP stimulation (ie, 33.49/µm²). However, the presence of dense-granule particles per se does not correlate entirely with the ability of the affinity-gold technique to label histamine, as there was no histamine labeling of the membrane-free, extruded granule particles that were focally attached to the cell surface during the degranulation phase induced by FMLP. Specificity controls (histamine absorption, DAO digestion) appropriately abrogated labeling with...
Fig 4. Human basophil granules in cells recovering from FMLP stimulation show altered, nearly empty (A), partially empty (B), and unaltered full granules (C and D) in the cytoplasm. (A and B) The altered granules are nearly devoid of granule matrix particles and contain central CLCs (c). The empty areas are largely devoid of DAO-G labeling, and the CLCs are not labeled for histamine. Cationized ferritin stains the overlying plasma membrane (A) but not the granule membranes, indicating that these are closed to the cell surface. (C) The granule is intensely labeled with DAO-G. Note that the gold labeling is attached only to the particulate matrix surrounding the central, unlabeled CLCs (c). (D) The unaltered granule is labeled with DAO-G, as is a particle-filled vesicle adjacent to it (arrow). (E) Poorly DAO-G–labeled granule particles, partially enwrapped by surface processes (arrows), are stained on their plasma membrane-associated side with cationized ferritin. Such images at late recovery time intervals suggest re-uptake of previously released, histamine-poor granule materials. OM: A, \( \times 59,000 \); B, \( \times 76,500 \); C, \( \times 68,000 \); D, \( \times 63,500 \); E, \( \times 88,500 \).

DAO-G of unaltered, electron-dense particle-filled basophil cytoplasmic secretory granules.

CLCs are present in basophil granules. These structures are homogeneous dense and assume typical bipyramidal shapes (or irregular, atypical shapes) in their intragranule locations in HBs. Using an ultrastructural immunogold technique, the sole protein constituent of CLCs (CLC protein) has been precisely localized to these intragranular structures. The enzyme-affinity–gold method to detect histamine (used in the current study) did not label CLCs in their intragranular locations or in their extracellular location, where they remain attached to basophil cell surfaces after extrusion from FMLP-stimulated cells. Thus, the major secretory granule of HBs is compartmental; it contains CLC protein within central crystals and histamine in the particle-rich granule matrix surrounding the central crystals. The CLCs that remained in altered, histamine-lacking granules in FMLP-stimulated cells (see below) also did not stain with DAO-G.

In samples obtained early after FMLP stimulation, altered granules were present in HBs undergoing PMD. Thus, in seconds after stimulation, focal pieces of granule materials are secreted, giving rise to focal electron lucencies within granule particles. Ultimately, the entire contents of granules can be secreted by a vesicular transport mechanism, but the granule membrane (container) is retained in the cytoplasm. These altered granules did not contain DAO-G histamine labeling exceeding background densities. Thus, it is certain that these altered granules no longer contain detectable histamine. The altered mast cell granules that occurred in quantity in the mast cell-rich inflammatory eye disease of interleukin-4 transgenic mice similarly had released their histamine content, as determined in vivo with the DAO-G stain. Thus, PMD of HBs in vitro and of mouse mast cells in vivo results in the emptying of granule containers, which remain morphologically intact but are devoid of histamine.

The FMLP-stimulated secretory continuum observed in HBs also resulted in the extrusion of granules and their
Fig 5. Reconstituted, unaltered, particle- and membrane-containing basophil cytoplasmic granules (G) in a cell recovered 6 hours after FMLP stimulation contain DAO-G labeling for histamine. N, nucleus; OM, × 45,000.

containers to the extracellular space. The resultant HBs were generally devoid of unaltered granules and altered granules within 1 to 2 minutes of stimulation, a time corresponding to biochemical detection of maximum histamine secretion. We have examined the standard ultrastructural morphology of granule reconstitution in HBs recovered 10 minutes to 6 hours after FMLP stimulation (A.M.D., unpublished data, January 1995). This process, while not complete in this early recovery study, did produce morphologic evidence of material and membrane conservation, granule content condensation, and synthesis. In the study reported here, DAO-G staining demonstrated that dense particle-filled, unaltered granules once again contained histamine, but that persistent extracellular granule materials did not. Intracytoplasmic granules, in the process of condensing dense materials within themselves, contained histamine, and focal accumulations of vesicles in the subplasma membrane and perigranular areas, as well as in expanded Golgi structures, also contained gold labeling for histamine. All such vesicular accumulations were absent at peak release times (1 to 2 minutes) in FMLP-stimulated cells. The vesicle collections in the recovering cells are similar to those in guinea pig basophils recovering from degranulation. Their histamine content, as described here, suggests two possible sources for the reconstitution of granular histamine in HBs: (1) re-uptake of previously released histamine, and (2) newly synthesized histamine in Golgi structures. As histamine from either source is reincorporated into granules, induction of granule content condensation would occur, analogous to the granule condensation properties of histamine.

Using DAO-G, we show that dense, unaltered granules of actively releasing HBs, as well as similar structures that are reconstituted after FMLP-stimulated degranulation, contain histamine, but that altered granules in stimulated cells undergoing degranulation are devoid of histamine before their membranes are extruded. Granule reconstitution is associated with DAO-G-positive cytoplasmic vesicles in the peripheral cytoplasm, near granules, and in Golgi areas. The appearance of these vesicles suggests transport to granules of histamine, either derived from new synthesis, re-uptake of released histamine, or both, and is associated with striking granule condensation of dense contents that contain histamine.

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