**RAPID COMMUNICATION**

**Vesicular Transport of Histamine in Stimulated Human Basophils**

By Ann M. Dvorak, Donald W. MacGlashan, Jr, Ellen S. Morgan, and Lawrence M. Lichtenstein

Human basophils participating in experimentally produced contact allergy display progressive secretion of electron-dense secretory granule contents and retention of cytoplasmic granule containers in the absence of entire granule extrusion, a process termed piecemeal degranulation (PMD) and postulated to be effected by vesicular transport (Dvorak HF, Dvorak AM: *Clin Hematol* 4:651, 1975). Proof of this hypothesis was sought using models of human basophil-stimulated secretion, partially purified human peripheral blood basophils, and a morphometric analysis of the fraction of total cellular cytoplasmic vesicles loaded with histamine, a major proinflammatory mediator present in basophil secretory granules. The subcellular localization of histamine was accomplished using a new ultrastructural enzyme-affinity-gold method based on the affinity of diamine oxidase for its substrate, histamine (Dvorak et al: *J Histochem Cytochem* 41:787, 1993). Two models were selected for a kinetic analysis of stimulated vesicle transport of histamine based on known biochemical and ultrastructural characteristics (MacGlashan et al: *J Immunol* 136:2231, 1986; Warner et al: *J Leukoc Biol* 45:558, 1989; Dvorak et al: *Am J Pathol* 141:1309, 1992; Dvorak et al: *Lab Invest* 64:234, 1991). These models were selected to include the rapid release reaction stimulated by the bacterial peptide, FMLP, and the slow release reaction stimulated by the phorbol diester tumor promoter, TPA. The results of this study showed that the fraction of histamine-loaded cytoplasmic vesicles (%VG/TV(μm³)) in TPA-stimulated basophils significantly exceeded the fraction in unstimulated cells, a process that persisted for 45 minutes after TPA stimulation and was associated with extensive PMD and no morphologic evidence of recovery. Similarly, the fraction of histamine-loaded cytoplasmic vesicles after FMLP stimulation significantly exceeded the fraction in unstimulated cells, a process that persisted for 10 minutes after FMLP stimulation and was associated with the morphologic continuum of PMD + anaphylactic degranulation (characterized by extrusion of granules) + recovery, a process largely complete in the 10-minute samples. These studies establish for the first time that an important proinflammatory mediator, histamine, traffics from secretory granules to the extracellular milieu in small cytoplasmic vesicles in stimulated human basophils. The association of this process with the ultrastructural release reaction defined as PMD produced primarily by TPA and in part by FMLP establishes vesicular transport as the mechanism for effecting this type of regulated secretion. Vesicular transport of histamine was also significant in the more complex stimulated secretory and recovery model produced by exposure of human basophils to the bacterial peptide FMLP.

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Submitted June 18, 1996; accepted September 4, 1996.

Supported by US Public Health Service Grant No. AI-33372.

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0006-4971/96/8811-0036$3.00/0

Blood, Vol 88, No 11 (December 1), 1996: pp 4090-4101
secretory granules could subserve a storage and rerelease function for circulating proteins in humans and animals.\textsuperscript{1,5,14-16} Reverse vesicular traffic of horseradish peroxidase was accomplished in washout experiments in the absence of granule release and resulting in peroxidase-negative intracytoplasmic granules, whereas stimulation of AND after granule loading with horseradish peroxidase resulted in extrusion of peroxidase-containing secretory granules.\textsuperscript{1}

Considerable progress has been accomplished regarding the identification of potential secretagogues for PMD by combining ultrastructural and biochemical kinetic studies of human basophil release reactions.\textsuperscript{17-22} These types of analyses have shown that some stimuli are associated primarily with PMD\textsuperscript{26,27} but that others stimulate a continuum of PMD \( \rightarrow \) AND with kinetics unique for individual stimuli.\textsuperscript{18} We have used two of these models\textsuperscript{18,19} with different anatomic and biochemical kinetics to do extensive studies of the transport of two materials in secretory human basophils. Two newly developed techniques to label these substances in small cytoplasmic vesicular structures were applied to the secretory models. These included the immunogold detection of Charcot-Leyden crystal (CLC) protein—a specific eosinophil and basophil protein\textsuperscript{21-23} (Dvorak et al, unpublished data, June 1996) and the enzyme-affinity gold detection of histamine,\textsuperscript{24,25} long regarded as a constituent of basophil (and mast cell) granules implicated in allergic inflammation.\textsuperscript{1,26-28} In the work reported here, we quantitated the proportion of total cytoplasmic vesicles that were labeled with diamine oxidase (DAO)-gold, indicating histamine,\textsuperscript{26} in human basophils stimulated with either tetradecanoyl phorbol acetate (TPA) or formyl methionyl leucyl phenylalanine (FMLP), and we made comparisons of these values to those of unstimulated cells, to different time points for each trigger, and between similar and disparate times for each secretagogue. We found that secretory human basophils move histamine in cytoplasmic vesicles and that the vesicular transport is similar to that suggested by morphologies and models previously recorded.\textsuperscript{1,18,19}

MATERIALS AND METHODS

Basophil purification. Buffy coat cells, obtained from normal donors undergoing hemapheresis, were partially purified by counter-current centrifugal elutriation and then placed on Percoll density gradients (1.075 and 1.066 g/mL). Basophils were recovered from the interface between the two Percoll layers.\textsuperscript{26} Two preparations, each containing 32% basophils, were used for these studies.

Histamine release. Cells were suspended in PAG CM (PIPES [piperazine N,N'-bis-2-ethane sulfonic acid]-albumin-glucose supplemented with 1 mM/L CaCl\textsubscript{2} and 1 mM/L MgCl\textsubscript{2}) and stimulated with either 100 ng/mL of the phorbol diester tumor promoter, 12-O-tetradecanoyl-phorbol 13-acetate (TPA)\textsuperscript{11,12} or with 1 mM/L of the bacterial peptide, FMLP,\textsuperscript{17,18} at 37°C for 30 or 60 minutes. The supernatant was removed and assayed for histamine release with an automated fluorometric technique.\textsuperscript{19} Buffer-incubated controls were also assayed, and spontaneous histamine release was subtracted to give final stimulated histamine release values. Histamine release was 79.1% and 66.2% at 60 minutes in each of two experiments after TPA stimulation and 33% at 30 minutes and 56% at 60 minutes after FMLP stimulation. The two secretagogues were used then based on detailed published documentation of the biochemistry and ultrastructural anatomy of their basophil release reactions that indicated a possible role for vesicular traffic,\textsuperscript{17,19,31,32} whereas similar initial studies of secretion induced by an IgE-mediated stimulus did not.\textsuperscript{24}

Electron microscopy. Aliquots of samples of cells stimulated with 100 ng/mL TPA (Sigma Chemical Co, St Louis, MO) or with 1 mM/L FMLP were recovered for electron microscopy at the following times poststimulus: for TPA—0 time, 1, 2, 5, 10, 15, 30, 45 minutes; for FMLP—0 time, 10, 20, 30 seconds, 1, 2, 5 and 10 minutes. Unstimulated buffer-incubated basophils (20 seconds, 1 and 10 minutes) were also prepared for electron microscopy as before.\textsuperscript{26} Briefly, cell suspensions were fixed by diluting them in a 10-fold excess of 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl\textsubscript{2} in 0.1 mM/L sodium cacodylate buffer, pH 7.4. Cells were fixed for 1 hour at room temperature, washed, and resuspended in 0.1 mM/L sodium cacodylate buffer, pH 7.4, 4°C. Some cell preparations were treated with cationized ferritin after fixation to stain cell surfaces, as previously done.\textsuperscript{26} Cell samples were suspended in warm 2% agar, rapidly centrifuged to form agar pellets containing cells, and postfixed for 2 hours at 4°C in 2% aqueous osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 mM/L sodium phosphate buffer, pH 6.0. Cell pellets were then dehydrated in a graded series of alcohols and embedded in a propylene oxide-Epon (Ted Pella, Inc, Redding, CA) sequence. Enzyme-affinity gold-stained thin sections (see next section) were stained with lead citrate and examined in a Philips 400 electron microscope (Philips Export B.V., Eindhoven, The Netherlands).

Enzyme-affinity procedure to demonstrate histamine. Thin sections of Epon-embedded material were prepared to demonstrate histamine with the ultrastructural enzyme-affinity method, based on diaminooxidase-gold (DAO-G) binding.\textsuperscript{26} Briefly, preparation of the DAO-G reagent was as follows: A colloidal suspension of gold was prepared according to the method of Frens.\textsuperscript{28} Four milliliters of an aqueous 1% solution of sodium citrate was added to a boiling aqueous solution of 100 mL 0.01% tetrachloraurvatic acid and allowed to boil for 5 minutes before cooling on ice. The pH of the colloidal gold suspension was adjusted to 7 with 0.2 mM/L potassium carbonate. Preparation of the DAO-G complex was according to the method of Bendayan.\textsuperscript{36} Three milligrams of DAO was dissolved in 0.3 mM distilled water and placed in a polycarbonate ultrafuge tube with 10 mL of the gold suspension. The mixture was centrifuged at 25,000 rpm for 30 minutes, 4°C, in a Beckman ultracentrifuge (Beckman Instruments, Inc, Palo Alto, CA). The DAO-G complex formed a red sediment that was carefully recovered and resuspended in 3 mL 0.1 mM/L phosphate-buffered saline (PBS) containing 0.02% polyethylene glycol, pH 7.6 (final concentration, 1 mg DAO/mL).

For cytochemical labeling, section-containing grids were inverted and floated on PBS drops for 5 minutes, followed by incubation on a drop of DAO-G at 37°C for 60 minutes. The grids were vigorously washed in distilled water and stained with dilute lead citrate for 10 minutes before viewing in a Philips 300 or 400 electron microscope. Specificity controls for the enzyme-affinity gold method included prior digestion of samples with diaminooxidase or absorption of the diaminooxidase-gold reagent by solid-phase histamine.

Ultrastructural data collection and analysis. One-micron sections of Epon blocks containing cell suspensions from each time point for each secretagogue, as well as for buffer-incubated controls, were prepared and viewed by light microscopy. Random thin sections of each block were made for the enzyme-affinity gold preparations, which were then viewed and sampled randomly by electron microscopy. Counts were done on 10 micrographs of basophils (and adjacent Epon background) imaged in 8 \( \times \) 10 prints at each interval after TPA or FMLP stimulation; this was also done with unstimulated basophils.

A total of 170 basophils comprised the morphometric sample. The total number of cytoplasmic, smooth membrane-bound vesicles and gold-labeled vesicles for each basophil was determined and expressed as the average number/\( \mu m^2 \) and as percent of total vesicles.
secretory granules were shown in unstimulated cells with an enzyme-affinity ultrastructural cytochemical technique. Granules that contained homogeneously dense CLCs within their particle matrix showed gold-labeled histamine only in the particulate matrix, not in the CLC. Specificity controls for the diamine-oxidase affinity-gold technique abrogated label for histamine when the DAO-G reagent was absorbed with histamine-bound agarose beads before staining sections (Fig 1C) or when sections were digested with diamine oxidase to remove granule histamine before staining with DAO-G (Fig 1D).

**RESULTS**

**Subcellular localization of histamine in human basophils.** Histamine-labeled, dense particle-packed human basophil secretory granules were shown in unstimulated cells with an enzyme-affinity ultrastructural cytochemical technique. Granules that contained homogeneously dense CLCs within their particle matrix showed gold-labeled histamine only in the particulate matrix, not in the CLC. Specificity controls for the diamine-oxidase affinity-gold technique abrogated label for histamine when the DAO-G reagent was absorbed with histamine-bound agarose beads before staining sections (Fig 1C) or when sections were digested with diamine oxidase to remove granule histamine before staining with DAO-G (Fig 1D).

**Vesicular transport of histamine in TPA-stimulated human basophils.** The ultrastructural changes in histamine-releasing human basophils that were induced by exposure to TPA and sampled at 0, 1, 2, 5, 10, 15, 30, and 45 minutes thereafter were similar to those previously reported. Briefly, the predominant secretory response of human basophils to TPA was PMD, which extended over the 45-minute kinetic interval tested. No evidence of recovery from secretion was evident during this time interval. Between 1 and 15 minutes, ultrastructural changes (termed a forme fruste of AND) appeared in a small number of cells. Characteristically, granule-plasma membrane fusions and release of granule contents without exteriorization of the granule containers occurred. By 30 minutes after exposure to TPA, a small number of cells showed classic AND with extrusion of granules and exteriorization of granule containers.

The mechanism for effecting PMD in human basophils has been postulated to be by vesicular transport of stored granule contents. We used a morphometric analysis of DAO-G-labeled, TPA-stimulated cells to quantitate this process here. Table 1 shows the average cell area, total number and average number of cytoplasmic vesicles, total number and average number of gold-labeled (histamine-carrying) vesicles, and the number of total vesicles/µm² and of gold-labeled vesicles/µm² for unstimulated, buffer-incubated cells (20 seconds, 1 and 10 minutes) as well as for TPA-stimulated cells collected at 0 time and 1, 2, 5, 10, 15, 30, and 45 minutes thereafter. During this kinetic sequence of changes stimulated in human basophils by TPA, the mean cell area did not differ significantly at individual times after stimulation compared with unstimulated cells (P = not significant [NS]), although a statistically insignificant trend toward

**Table 1. Total Cytoplasmic Vesicles and Histamine-Loaded Vesicles in TPA-Activated Human Basophils**

<table>
<thead>
<tr>
<th>TPA</th>
<th>Average Cell Area ± SE</th>
<th>Total No.</th>
<th>Average No. ± SE</th>
<th>Total Vesicles/µm² ± SE</th>
<th>Total Gold-Labelled Vesicles/µm² ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.48 ± 1.77</td>
<td>393</td>
<td>39.3 ± 3.29</td>
<td>1.82 ± 0.18</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>0 time</td>
<td>24.15 ± 1.29</td>
<td>309</td>
<td>30.9 ± 4.37</td>
<td>1.28 ± 0.16</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>1 min</td>
<td>28.36 ± 3.44</td>
<td>353</td>
<td>35.3 ± 4.56</td>
<td>1.39 ± 0.23</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>2 min</td>
<td>20.38 ± 1.11</td>
<td>280</td>
<td>27.0 ± 3.43</td>
<td>1.33 ± 0.18</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>5 min</td>
<td>22.67 ± 1.25</td>
<td>193</td>
<td>19.3 ± 3.98</td>
<td>0.93 ± 0.27</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>10 min</td>
<td>21.91 ± 1.33</td>
<td>334</td>
<td>34.4 ± 3.75</td>
<td>1.60 ± 0.22</td>
<td>0.69 ± 0.12</td>
</tr>
<tr>
<td>15 min</td>
<td>22.81 ± 0.96</td>
<td>199</td>
<td>19.9 ± 4.15</td>
<td>0.91 ± 0.22</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>30 min</td>
<td>26.24 ± 1.37</td>
<td>137</td>
<td>13.8 ± 1.36</td>
<td>0.56 ± 0.06</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>45 min</td>
<td>16.19 ± 1.51</td>
<td>155</td>
<td>15.5 ± 2.27</td>
<td>0.97 ± 0.10</td>
<td>0.53 ± 0.06</td>
</tr>
</tbody>
</table>

that were gold-labeled (% VGN). Statistical comparisons were made using the Kruskall-Wallis nonparametric ANOVA and χ² tests.
smaller cells in the 45-minute sample (16.19 μm) compared with unstimulated cells (22.48 μm) was present (P = NS) (Table 1). From these data, the fraction of available total cellular vesicles carrying histamine (gold-labeled) was calculated (Table 2, Fig 2).

The percent of VG/Tv was similar in unstimulated cells and in TPA-stimulated cells obtained at time 0 (P = NS). At 1 minute stimulation with TPA, 30% of the available vesicles were gold-labeled (P < .025 compared with unstimulated cells, 22%); at 2 minutes, 29% were gold-labeled (P < .05, compared with unstimulated cells); at 10 minutes, 45%; 15 minutes, 56%; 30 minutes, 39%; and at 45 minutes, 55%; values all significantly greater than unstimulated cells (P < .001). Thus, the vesicles carrying histamine in TPA-stimulated cells increased and remained elevated in the same time frame during which histamine release was measured (79.1%, 66.2% at 60 minutes in each of two experiments after TPA stimulation). Additional significance values for inter-sample comparisons are listed in Table 2. For example, all stimulated samples from 10 minutes on had significantly more VG/Tv than at 0 time (P < .001); the 5- (P < .025), 10- and 15- (P < .001), 30- (P < .05), and 45-minute (P < .001) samples exceeded the one-minute sample; the 10-, 15- (P < .001), 30- (P < .05), and 45-minute (P < .001) samples exceeded the 2-minute sample; all samples from 10 minutes on (P < .001) exceeded the 5-minute sample; the 15-minute (P < .025) and 45-minute (P < .05) samples exceeded the 10-minute sample; the 30-minute sample (P < .025) was less than the 15-minute sample; the 45-minute (P < .01) sample exceeded the 30-minute sample. Thus, progressive significant increases in histamine-carrying vesicles occurred in TPA-stimulated cells concomitant with the development and persistence of PMD without evidence of recovery of basophils either by synthetic or endocytotic mechanisms or by a mixture of these processes.25,38

Ultrastructural identification of histamine-labeled cytoplasmic vesicles was possible using the DAO-G enzyme-affinity technique (Fig 3). At all times after exposure to TPA, it was possible to identify gold-loaded cytoplasmic vesicles primarily in the perigranular and peripheral cytoplasmic areas. These vesicles were bounded by smooth membranes and displayed either electron-lucent interiors or contained electron-dense particles analogous to the particles filling the much larger secretory granules. Some gold-labeled electron-lucent vesicles were surrounded by masses of glycogen (Fig 3B). Specificity controls showed abrogation of label in vesicles and granules (data not shown). Rarely, fusion of gold-labeled vesicles with the plasma membrane was visible (Fig 3G). Gold-loaded, perigranular cytoplasmic vesicles adjacent to particle-packed granules (Fig 3C, E, and F) as well as adjacent to empty granules devoid of their electron-dense contents (Fig 3H and L) were noted, persisting to the 45-minute time interval after TPA activation (Fig 3L).

Vesicular transport of histamine in f-Met peptide-stimulated human basophils. The ultrastructural changes in histamine-releasing human basophils that were induced by exposure to FMLP and sampled at 0, 10, 20, and 30 seconds and 1, 2, 5, and 10 minutes thereafter were similar to those previously reported.18 Briefly, FMLP stimulated a rapid continuum of secretory and recovery responses in the 10-minute study interval corresponding to the biochemical kinetics of mediator (histamine) release.17,18 This continuum included an early PMD phase, followed by an AND phase with cytoplasmic granule recovery by 10 minutes in these samples. The majority of cells participated in this anatomic continuum.
Fig 3. TPA-stimulated human basophils prepared with DAO-gold to detect histamine studied at 0 time (A), 2 minutes (B through D), 5 minutes (E through G), 10 minutes (H and I), 15 minutes (J), 30 minutes (K), and 45 minutes (L) after activation. In (A), at 0 time, the basophil granule (g) is labeled with DAO-G. In (B), at 2 minutes, three cytoplasmic vesicles near the cell surface (open arrowhead) are labeled for histamine. One gold-labeled vesicle is electron-lucent and is encased in electron-dense glycogen particles (closed arrowhead). Another gold-labeled vesicle also contains granule particles (long arrow) and a third gold-labeled vesicle is electron-lucent (short arrow). In (C), at 2 minutes, note the gold-labeled vesicle filled with granule particles adjacent to the labeled granule (g). In (D), also at 2 minutes, the electron-lucent vesicle contains DAO-gold. In (E through G), at 5 minutes, the cell surface is indicated by open arrowheads. Note in panels (E) and (F) the granule particle-filled, DAO-gold-labeled perigranular vesicles (open arrows) adjacent to the cell surfaces. The adjacent granules (g) also label for histamine; the granule in (F) has a focal, electron-lucent region (arrow) typical for PMD. Cytoplasmic glycogen particles (arrows in E) are electron-dense and generally larger than the ~20-nm gold label. In (G), at 5 minutes, a DAO-gold-labeled vesicle is fused to the cell surface (open arrowhead). In (H) and (I), at 10 minutes, electron-lucent (H) and particle-filled (I) cytoplasmic vesicles (arrows) contain histamine. In (H), a large empty granule (cc) devoid of gold label and granule particles is typical for PMD. In (I), the cell surface is coated with cationized ferritin (used in cell processing for electron microscopy) and is indicated by the open arrow. The granule particle–filled cytoplasmic vesicles are labeled with DAO-gold (arrows) at 15 (J), 30 (K), and 45 (L) minutes after TPA activation. The cell surface is indicated by open arrowheads in (K) and (L); an empty granule (cc) typical for PMD is present at 45 minutes poststimulation in (L). OMs: (A and B), \times 55,000; (C), \times 117,500; (D), \times 69,000; (E and K), \times 71,500; (F), \times 61,000; (G), \times 80,000; (H), \times 67,500; (I and J), \times 113,000; (L), \times 54,000.

The relationship of cytoplasmic vesicles and their contents to the complex secretory response stimulated in human basophils by FMLP was examined using a morphometric analysis of DAO-G-labeled cells. Table 3 shows the average cell area, total and average numbers of cytoplasmic vesicles, total and average numbers of gold-labeled (histamine-carrying) vesicles, and the numbers of total and gold-labeled vesicles/μm² for unstimulated, buffer-incubated cells (20 seconds, 1
and 10 minutes) as well as for FMLP-stimulated cells collected at 0 time and at 10, 20, and 30 seconds and 1, 2, 5, and 10 minutes thereafter. During this kinetic sequence of changes stimulated in human basophils by FMLP, the mean cell area did not differ significantly at individual times after stimulation compared with unstimulated cells (P = NS). From these data, the fraction of available total cellular vesicles carrying histamine (gold-labeled) was calculated (Table 4, Fig 4).

The percent of VG/TV/μm² was significantly elevated in all FMLP-stimulated samples compared with unstimulated cells (P < .001) (Fig 4). Thus, the vesicles carrying histamine in FMLP-stimulated cells increased and remained elevated during the same time frame in which histamine release can be measured and in which anatomic recovery of histamine-secreting cells takes place. Significance values for inter-sample comparisons are also listed in Table 4. These comparisons showed that the 10-, 20-second (P < .001), 30-second (P < .005), 1-minute (P < .025), and 5-minute (P < .001) samples all exceeded the 0 time point of stimulated cells; the 20-second (P < .001), 30-second (P < .005), and 1-, 2-, 5-, and 10-minute (P < .001) samples all exceeded the 10-second sample; the 30-second (P < .001), 2-minute (P < .025), and 10-minute (P < .001) samples exceeded the 20-second sample; the 1-minute (P < .001), 2-minute (P < .005) and 5-minute (P < .001) samples exceeded the 30-second sample; the 10-minute (P < .005) sample exceeded the 1-minute sample; the 5-minute (P < .025) sample exceeded the 2-minute sample, and the 10-minute (P < .001) sample exceeded the 5-minute sample. Thus, progressive significant increases in histamine-carrying vesicles occurred in FMLP-stimulated cells concomitant with the development of the anatomic secretory continuum PMD → AND → recovery. Recovery mechanisms for human basophils after FMLP stimulation include vesicular trafficking of synthetic and endocytotic materials.

Ultrastructural identification of histamine-labeled cytoplasmic vesicles was done using the DAO-G technique (Fig 5). Gold-labeled vesicles were present in all samples of FMLP-stimulated cells. The structures were smooth membrane-bound and either displayed an electron-lucent interior or contained dense particles identical to those in the much larger secretory granules. In addition to the presence of these gold-labeled vesicles in the perigranular and peripheral cytoplasmic areas, and in contrast to TPA-activated cells, some were also present in the cytoplasm containing Golgi structures. Some gold-labeled vesicles rested beneath the plasma membrane, through which granules had been extruded during the AND phase of the degranulation continuum (Fig 5K). The FMLP-stimulated samples contained particle-filled granules that labeled for histamine (Fig 5E and F); these samples also contained granules (partially and completely devoid of electron-dense materials) with reduced or absent histamine stores (Fig 5C and F). Specificity controls for the DAO-G technique in these samples showed abrogation of granule and vesicle label when the DAO-G reagent was absorbed by histamine beads before staining or when DAO digestion of sections was done before DAO-G staining (Fig 5D and G).

<table>
<thead>
<tr>
<th>Time</th>
<th>%VG/TV/μm²</th>
<th>Significance To</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 time</td>
<td>22</td>
<td>UN</td>
</tr>
<tr>
<td>10 s</td>
<td>39</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>20 s</td>
<td>72</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>30 s</td>
<td>49</td>
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<tr>
<td>1 min</td>
<td>67</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>2 min</td>
<td>62</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>5 min</td>
<td>72</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>10 min</td>
<td>56</td>
<td>P &lt; .001</td>
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Table 3. Total Cytoplasmic Vesicles and Histamine-Loaded Vesicles in FMLP-Activated Human Basophils

<table>
<thead>
<tr>
<th>FMLP Stimulation Time</th>
<th>Total Vesicles</th>
<th>Gold-Labeled Vesicles</th>
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<tbody>
<tr>
<td></td>
<td>Average No. SE</td>
<td>10 s No. SE</td>
</tr>
<tr>
<td>Control</td>
<td>22.48 1.77</td>
<td>393 3.29</td>
</tr>
<tr>
<td>0 time</td>
<td>27.69 1.24</td>
<td>415 5.10</td>
</tr>
<tr>
<td>10 s</td>
<td>28.24 1.37</td>
<td>405 5.13</td>
</tr>
<tr>
<td>20 s</td>
<td>21.10 0.69</td>
<td>471 5.01</td>
</tr>
<tr>
<td>30 s</td>
<td>24.37 1.64</td>
<td>447 6.84</td>
</tr>
<tr>
<td>1 min</td>
<td>21.56 1.76</td>
<td>307 2.63</td>
</tr>
<tr>
<td>2 min</td>
<td>18.21 2.00</td>
<td>158 3.81</td>
</tr>
<tr>
<td>5 min</td>
<td>26.73 1.61</td>
<td>276 28.7</td>
</tr>
<tr>
<td>10 min</td>
<td>22.64 1.01</td>
<td>156 2.52</td>
</tr>
</tbody>
</table>

Table 4. Histamine-Loaded Cytoplasmic Vesicles in FMLP-Activated Human Basophils
Cytoplasmic vesicles attached by fusion to large secretory granules were evident in the 0 time FMLP-stimulated sample (Fig 5C). These fused vesicles were attached to virtually empty granules with reduced gold label (Fig 5C) as well as to granules with particles and gold-labeled histamine (Fig 5E). Some cytoplasmic vesicles containing gold label for histamine were surrounded by glycogen aggregates (Fig 5C, inset).

Vesicular transport of histamine in the basophils of the same donors has different kinetics, analogous to biochemically measured release of histamine, when TPA and FMLP activation are compared. In Table 5, comparisons of the %VG/TV/µm² are listed for similar (and several disparate) times tested when the basophils from the same donors were stimulated by either TPA (slow kinetics) or FMLP (fast kinetics). Significantly larger numbers of total vesicles were gold-loaded in the FMLP-triggered cells than in the TPA-triggered cells (P < .001 for 0 time, 1-, 2-, and 5-minute samples, and P < .01 for the 10-minute samples (Fig 6). Thus, a secretagogue that rapidly induced the anatomic continuum of PMD → AND → recovery also induced more histamine-loaded vesicles, compared with similar times after stimulation, than a secretagogue which slowly induced PMD, persisting to involve ~50% of the cells by 45 minutes and showing only minor amounts of AND and no morphologic evidence of recovery.

Comparison of the fraction of total vesicles carrying histamine at disparate times after stimulation with TPA or FMLP was also of interest (Table 5). For example, the peak value for the %VG/TV/µm² for TPA was at 15 minutes. This value (56%) was significantly less than the peak value (72%) for FMLP, which occurred at 20 seconds after stimulation (P < .001). TPA stimulation induced elevated VG/TV/µm² at 45 minutes (55%) in samples predominantly still undergoing PMD (and with small amounts of AND and with no anatomic signs of recovery), whereas predominantly recovering cells (studied 5 minutes after FMLP stimulation) had significantly more VG/TV/µm² (72%; P < .001). End times of kinetic samples for each secretagogue showed no significant difference in the %VG/TV/µm² (TPA, 45 minutes 55% vs. FMLP, 10 minutes 56%, P = NS).

DISCUSSION

We have used a new enzyme-affinity gold method to localize histamine in subcellular organelles to quantitate the number of cytoplasmic vesicles in secreting human basophils that contain this pro-inflammatory mediator. By examining multiple time points after stimulation with two different secretagogues known to have different biochemical histamine release and morphologic kinetics, we have compared the proportion of total vesicles transporting histamine with those in unstimulated cells, as well as between different times after exposure to an individual secretagogue, and between identical, or disparate, times after exposure to each of two

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**Fig 4.** Percentage of total cytoplasmic vesicles that are labeled with gold, indicating the presence of histamine in unstimulated human basophils and in human basophils stimulated with FMLP (0 to 10 minutes). *P < .001 compared with unstimulated cells.

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**Fig 5.** FMLP-stimulated human basophils prepared with DAO-gold to detect histamine (A through C, E, F, H through L) or with specificity controls (D and G) at 0 time (A through D), 10 seconds (E through G), 20 seconds (H), 30 seconds (I), 1 minute (J and K), and 10 minutes (L) after activation. The open arrowhead in all panels indicates the cell surface; g, granule, eq, empty granule (typical for PMD); n, nucleus. At 0 time and in the same activated cell, both empty, electron-lucent (arrow in A) and full, electron-dense (arrow in B) vesicles are gold-labeled. Cytoplasmic glycogen particles are electron-dense (A). In (C), a nearly empty granule (typical for PMD) retains some DAO-gold label. Some cytoplasmic vesicles containing gold label for histamine were surrounded by glycogen aggregates (Fig 5C, inset).

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DISCUSSION

We have used a new enzyme-affinity gold method to localize histamine in subcellular organelles to quantitate the number of cytoplasmic vesicles in secreting human basophils that contain this pro-inflammatory mediator. By examining multiple time points after stimulation with two different secretagogues known to have different biochemical histamine release and morphologic kinetics, we have compared the proportion of total vesicles transporting histamine with those in unstimulated cells, as well as between different times after exposure to an individual secretagogue, and between identical, or disparate, times after exposure to each of two
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secretogogues. Similar to previous reports, DAO-gold labeled histamine in human basophil secretory granules and cytoplasmic vesicles; cytoplasmic granules in activated cells partially or completely devoid of electron-dense matrix had reduced or absent histamine stores.

The phorbol ester, TPA, and the bacterial peptide, FMLP, each stimulate the release of pro-inflammatory mediators from human basophils; the kinetics and products of release are unique, however, for each secretogogue. Generally, the release of histamine from FMLP-stimulated human basophils is extremely rapid and essentially complete in ~2 to 3 minutes. The major product of arachidonic acid metabolism
produced by human basophils, leukotriene C₄ (LTC₄), lags behind histamine release, being complete for this secretogogue in ~7 minutes post-stimulus.¹⁷,⁴⁰ TPA-stimulated human basophils do not generate LTC₄ at all and release histamine slowly (maximum release ~1 hour after stimulation).¹⁷,¹⁹,⁴⁰ These unique biochemical characteristics for release responses stimulated by FMLP and TPA are accompanied by characteristic, unique morphologies for each trigger, as determined by routine electron microscopy.¹⁸,¹⁹ Multiple samples examined after FMLP stimulation, for example, showed a rapid continuum of changes which proceeded from PMD → AND → recovery, a process essentially complete by 10 minutes.¹⁸ In contrast, multiple samples examined after TPA stimulation showed extensive PMD extending over a 45-minute interval and involving ~50% of the granules in cells by that time.¹⁹ To a small extent, typical AND was present and unique interactions of granule and plasma membranes also occurred with TPA stimulation—a process termed forme fruste of AND. In the latter case, granule chambers fused to plasma membranes and emptied their contents, but the chamber membranes did not evert. No morphologic evidence of granule recovery occurred at 45 minutes after TPA activation. These two well-described models of disparate biochemical and morphological human basophil release reactions were used in the present study to examine the hypothesis that cytoplasmic vesicle transport of histamine could be stimulated (eg, regulated secretion) and that this transport system proceeded from granule to plasma membranes as a mechanism for effecting PMD.

The principal findings and interpretations of the specific enzyme-affinity gold labeling of cytoplasmic vesicles in secreting human basophils stimulated by TPA in the current study are as follows: (1) the fraction of gold-labeled vesicles (indicating histamine) of the total vesicles was elevated in all stimulated samples compared with unstimulated samples (thus, histamine is transported in vesicles in cells undergoing PMD and releasing histamine); (2) comparison of timed samples show a progressive increase in gold-labeled vesicles over time persisting to 45 minutes, except in the 30-minute sample. The value at 45 minutes was equivalent to the 10-minute sample and was significantly less than that at 15 minutes. Thus, as histamine release increased and persisted and PMD developed, so did the transport of histamine-loaded vesicles. AND began in the 30-minute sample, and basophils revealing this anatomy had diminished cytoplasmic vesicles.²⁵,⁴¹ However, the proportion of these vesicles carrying histamine at 30 minutes still significantly exceeded values for controls and early samples (eg, 0 to 5 minutes).

The principal findings and interpretations for the DAO-gold labeling of cytoplasmic vesicles in secreting human basophils stimulated by FMLP are as follows: (1) histamine-labeled vesicles in all stimulated samples exceeded those in unstimulated samples (thus, histamine is transported in vesicles in cells releasing histamine and undergoing PMD → AND → recovery); (2) comparisons of timed samples generally revealed increases in later samples compared with earlier samples. For example, all samples between 20 seconds and 10 minutes exceeded the 10-second value. The increased VG/TV indicating histamine transport was accompanied primarily by the morphology of PMD at early times (10 seconds to 1 minute), indicating stimulated secretory transport of vesicle-packaged histamine. At later times (2 to 10 minutes), the increased VG/TV was associated with the morphology of recovery of granule contents, indicating transport of vesicle-packaged histamine from two possible sources (endocytosis of released histamine, synthesis in Golgi structures) as mechanisms for effecting granule content reconstitution.

Quantitative comparisons of the fraction of total cytoplasmic vesicles loaded with histamine in these two models of human basophil activation were interesting. Because the samples for each secretogogue were aliquots of peripheral blood basophils purified from the same donors and on the same days, interindividual donor and daily variation in secretory response was eliminated in this analysis. The principal findings and interpretations of these comparisons are as fol-

### Table 5. Comparisons of Histamine-Loaded Vesicles in Human Basophils From the Same Donors Stimulated With TPA or FMLP

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
<th>Stimulus</th>
<th>%VG/TV/μm²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>TPA</td>
<td>0</td>
<td>24</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>0</td>
<td>FMLP</td>
<td>0</td>
<td>59</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>1 min</td>
<td>TPA</td>
<td>1 min</td>
<td>39</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>1 min</td>
<td>FMLP</td>
<td>1 min</td>
<td>67</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>2 min</td>
<td>TPA</td>
<td>2 min</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>FMLP</td>
<td>2 min</td>
<td>62</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>5 min</td>
<td>TPA</td>
<td>5 min</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>FMLP</td>
<td>5 min</td>
<td>72</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>10 min</td>
<td>TPA</td>
<td>10 min</td>
<td>45</td>
<td>P &lt; .01</td>
</tr>
<tr>
<td>10 min</td>
<td>FMLP</td>
<td>10 min</td>
<td>56</td>
<td>P = NS</td>
</tr>
<tr>
<td>20 s</td>
<td>FMLP</td>
<td>20 s</td>
<td>72</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>45 min</td>
<td>TPA</td>
<td>45 min</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>45 min</td>
<td>FMLP</td>
<td>45 min</td>
<td>72</td>
<td>P &lt; .001</td>
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<tr>
<td>45 min</td>
<td>FMLP</td>
<td>45 min</td>
<td>55</td>
<td></td>
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<tr>
<td>10 min</td>
<td>FMLP</td>
<td>10 min</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

![Graph](image_url)  
Fig 6. Percentage of total cytoplasmic vesicles that are labeled with gold, indicating the presence of histamine in unstimulated human basophils and in human basophils stimulated with FMLP (●) or TPA (●) for similar times. *P < .01; **P < .001 for comparisons between stimuli at similar times.)
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lows: (1) at identical times poststimulation (eg, 0 time, 1, 2, 5, and 10 minutes), the fraction of VG/TV stimulated by FMLP exceeded that stimulated by TPA (ie, the rapid secretory event characterized by the morphological continuum PMD \( \rightarrow \) AND \( \rightarrow \) recovery [FMLP] was associated generally with more histamine-loaded vesicular traffic than was the slow secretory event characterized by persistent PMD, a forme fruste of AND and no recovery [TPA]); (2) the peak value of %VG/TV for the slow trigger (TPA) at 15 minutes (56%) was less than that value for the fast trigger (FMLP) at 20 seconds (72%) (thus, speed of secretion was associated with greater histamine-loaded vesicular traffic at peak histamine-loaded vesicle times); (3) at 45 minutes after TPA stimulation, fewer TV were carrying histamine (55%) than at 5 minutes after FMLP stimulation (73%) (thus, continuing PMD and no recovery is associated with less traffic of histamine in vesicles than in samples displaying extensive and ongoing morphological evidence of recovery, as seen at 5 minutes after FMLP stimulation); (4) the percent of VGTV was identical in the end time samples for each stimulant of human basophil secretion—eg, TPA, 45 minutes, 55% and FMLP, 10 minutes, 56%, \( P = NS \) (thus, elevated histamine vesicular traffic [over unstimulated cells] persists in cells that are still secreting by PMD but not recovering [eg, TPA stimulation] as well as in cells that are recovering granule materials by endocytosis and synthesis, but are no longer secreting by PMD [eg, FMLP stimulation]).

The ability to correlate directly the fraction of histamine-loaded vesicles (%VG/V) in human basophils, as determined with this new ultrastructural imaging technique, to the biochemical measurements of histamine release in the supernatants of challenged cells is problematic. To do so with reasonable assurance that these data may be correlated, one would like to know the relative biochemical concentrations of histamine in granules and vesicles.

The enzyme cytochemical method, by definition a postembedding ultrastructural label, only accesses histamine at the cut surfaces of vesicles and granules and not throughout these organelles. Therefore, the real histamine content of these structures is not discernible with this method and, therefore, their relative contributions to the released histamine measured biochemically are not known. Also, to compare directly the cytochemical data with the histamine release data for each of two triggers with vastly different morphologies and kinetics, reflecting the mechanics of secretion, is difficult. For example, the slow-release model (TPA) essentially shows higher histamine release values (but lower histamine-loaded vesicles) than the rapid-release model (FMLP). A possible interpretation of these apparently disparate data is that, in the TPA model, the efficiency of vesicular transport of histamine that is still ongoing at 45 minutes (and in the absence of any recovery from secretion) contributes significantly to the higher biochemical values for histamine release. In the FMLP model, histamine secretion (as determined biochemically) might be less than that in the TPA model because the entire secretion-recovery sequence takes place rapidly. That is, the larger percent VG/V in the PMD phase of this secretory continuum evolves into a definitive granule extrusion phase with subsequent recovery. However, these interpretations must remain hypothetical until direct biochemical measurements of the histamine content in individual cytoplasmic vesicles and granules of basophils are possible.

Basophils of multiple species are characterized by numerous small, cytoplasmic, smooth membrane-bound vesicles.\(^5\) These structures are uniquely present in the perigranular and subplasma membrane cytosol in activated cells, and ultrastructural studies have detected similar vesicles attached to granules and fused to plasma membranes. Glycogen particles and aggregates are frequently closely associated with these vesicles and have been proposed as a possible energy source for their transport.\(^5\) These images prompted a generic degranulation model for basophils and mast cells to be put forth in 1975.\(^5\) Simply stated, this model proposed a continuum of secretion, encompassing PMD \( \rightarrow \) AND, mediated by vesicular transport of granule materials out of cells closely coupled to endocytotic vesicular traffic into cells. This coupled stimulus-secretion model also proposed that the morphologic expression of PMD or AND depended on the rate of vesicular traffic in that, at sufficiently slow rates, individual vesicles would traffic individually while exchanging granular contents, but that, as the traffic rate increased, discrete vesicles no longer would retain their individuality and would fuse to form channels connected to adjacent granules and to the plasma membrane, thereby creating the anatomy of AND. (An appropriate analogy would be the rate of water flow from a faucet which can be regulated to produce individual drops at slow speed, whereas fusion of drops would produce a stream of water at fast speed.)

The studies reported here support this model of basophil degranulation. For example, the slow rate of secretion of histamine stimulated by TPA was accompanied primarily by the morphology of PMD, effected by vesicular traffic of individual discrete vesicles loaded with histamine; little evidence of the morphology of AND occurred in this slow-secretion model. On the other hand, extremely rapid secretion of histamine, stimulated by FMLP, was accompanied by the morphology of PMD, effected by vesicular traffic at early times, and followed by the morphology of AND, with reduced numbers of vesicles.

Several recent studies of cytochemical and immunogold-labeled vesicles in stimulated secretory cells also support the use of these small containers in secretion from granulated secretory cells.\(^9\) The secreted materials packaged in transport vesicles include eosinophil peroxidase in interleukin-5–stimulated human eosinophilic myelocytes\(^9\) and mature eosinophils,\(^10\) amylase in norepinephrine-stimulated, isolated rat parotid gland acinar cells,\(^11\) CLC protein in FMLP- (Dvorak et al, unpublished data, June 1996) and TPA-\(^12\) stimulated human basophils, and histamine in FMLP-stimulated human basophils.\(^13\) Together with the new studies we report here, the transport of large granule secretory contents in small vesicular containers during stimulated secretion from inflammatory cells (eosinophils, basophils) and exocrine secretory cells (parotid gland acinar cells) is established.

REFERENCES


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