

Diamine oxidase–gold enzyme-affinity ultrastructural demonstration that human gut mucosal mast cells secrete histamine by piecemeal degranulation in vivo

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Biopsy specimens of human ilea were prepared for the ultrastructural detection of histamine in vivo with a new enzyme-affinity method (Dvorak et al., *J Histochem Cytochem* 1993; 41:787-800). Human gut mucosal mast cells contained histamine in cytoplasmic secretory granules that were electron dense, regardless of underlying substructural patterns. Partially and completely empty cytoplasmic granules, characteristic for piecemeal degranulation (Dvorak et al., *Int Arch Allergy Immunol* 1992;99:74-83) had diminished and absent histamine stores, whereas electron-dense granules in the same cells retained their histamine. Interstitial collagen (but not basal lamina of other cells) near secretory mast cells avidly bound histamine, thereby providing a potential source for re-release of extracellular histamine stores. These studies provide the first documentation of histamine secretion in vivo during piecemeal degranulation of human mast cells. (*J Allergy Clin Immunol* 1997;99:812-20.)

Key words: Mast cells, diamine oxidase–gold, histamine, piecemeal degranulation, human inflammatory bowel disease, histaminase, collagen, secretion, extracellular matrix

Ultrastructural studies of human gut mast cells (HGMCs) in vivo identified a secretory mechanism (distinct from classic regulated secretion by granule extrusion) termed *piecemeal degranulation* (PMD).¹ This anatomic process is distinctive and is recognized by the retention of partially filled and empty secretory containers in undamaged cells that also contain typical, electron-dense mast cell granules. Cells that are completely degranulated retain their empty containers: in wound healing, mast cells that are relieved of their granule contents by PMD, recover by replenishing these containers.²

PMD, of HGMCs in vivo was first described in patients with Crohn's disease (CD), an inflammatory bowel disease of unknown cause.³ Since the initial recognition of this form of secretion in human mast cells, extensive ultrastructural studies have clearly indicated that this form of secretion is the most prevalent type in mast cells in human

Abbreviations used

CD:	Crohn's disease
DAO:	Diamine oxidase
DAO-G:	Diamine oxidase–gold
FP:	Familial polyposis
HGMCs:	Human gut mast cells
PMD:	Piecemeal degranulation
UC:	Ulcerative colitis

diseases (reviewed in Dvorak⁴). Also, numerous studies now implicate mast cell biologic processes in the pathogenesis of inflammatory bowel diseases.⁵⁻⁹

Recently, we developed a new ultrastructural enzyme-affinity gold method to detect histamine in well-preserved, routinely prepared electron microscopic samples.¹⁰ The method is based on the affinity of diamine oxidase (DAO, i.e., histaminase) for its substrate, histamine, which is detected by visualizing the electron-dense gold label attached to DAO. Extensive specificity studies have validated the DAO-gold (DAO-G) method (in the detection of histamine) with the use of isolated, purified human lung mast cells.¹⁰ Application of this method to mast cell-rich, symptomatic inflammatory eyelid disease, which develops in a transgenic IL-4 mouse model, revealed that there was extensive PMD of mast cells in the eyelid lesions and that histamine had been secreted from the remaining, empty mast cell granule containers.¹¹ Thus the ultrastructural intracellular location of mast cell histamine and its secretion in vivo was first documented.¹¹

We applied the DAO-G method to examine HGMCs in vivo, using tissues from patients with ulcerative colitis (UC), CD, and familial polyposis (FP), which are conditions known to express PMD of mast cells.¹ In this report, we demonstrate that HGMCs undergoing PMD in vivo in these tissues also secrete histamine from their granule containers and thus document the ultrastructural mechanics of histamine secretion from human mast cells in gut tissues in vivo.

METHODS

Case material

Biopsy specimens of ileal mucosa from either conventional ileostomies or ileal or pelvic continent pouches were obtained

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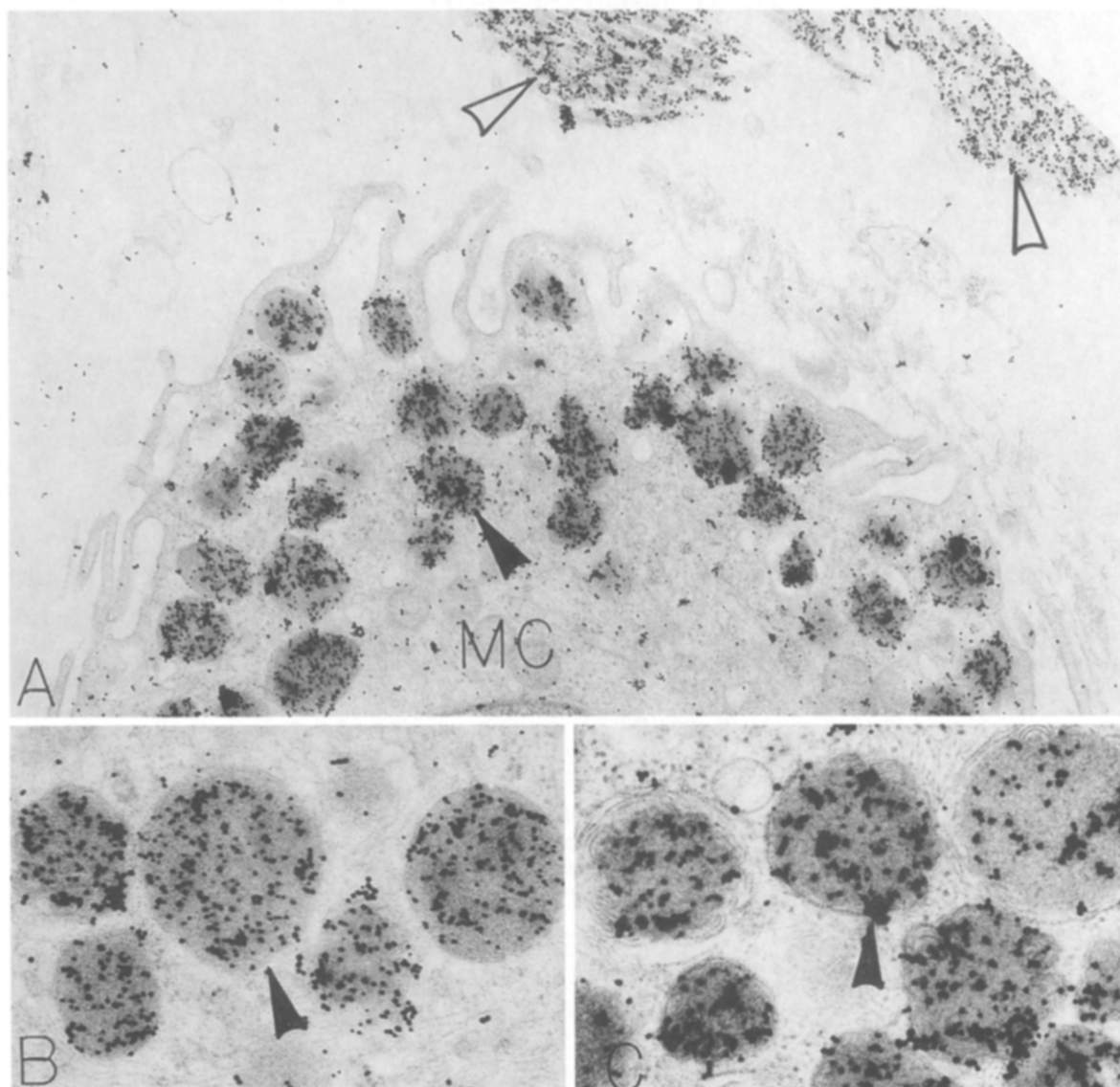


FIG. 1. Histamine is indicated by extensive gold label over electron-dense granules (closed arrowheads) of nonsecretory mast cells (MC) and bound to nearby interstitial collagen bundles (open arrowheads). **A**, Original magnification $\times 22,000$; **B**, $\times 43,000$; **C**, $\times 63,000$.

from patients with either inflammatory bowel disease (UC, CD) or FP, as described.^{1, 12-16} Ten cases were selected for study by ultrastructural enzyme-affinity cytochemistry to demonstrate histamine¹⁰ on the basis of prior knowledge of the ultrastructural pathologic features documenting the presence of mucosal mast cell degranulation.¹ In the 10 cases examined, the site of biopsy was the ileal pouch ($n = 6$) or ileal stoma ($n = 4$). The basic disease was CD ($n = 1$), UC ($n = 8$), or FP ($n = 1$). Samples were obtained either from acutely inflamed tissues (pouchitis, $n = 2$) or normal-appearing mucosa ($n = 8$), as determined at endoscopic examination. Of the 8 cases with endoscopically noninflamed mucosa, 4 were obtained from continent ileal pouches and 4 from conventional ileostomies. Bacterial invasion of ileal

biopsy specimens was found by ultrastructural determination ($n = 4$) or anaerobic cultures ($n = 7$).¹²

Processing for routine electron microscopy

Tissue biopsy specimens were fixed by immersion in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl_2 in 0.1 mol/L sodium cacodylate buffer at a pH of 7.4. Samples were fixed for 2 hours at room temperature, washed, and resuspended in 0.1 mol/L sodium cacodylate buffer at a pH of 7.4 at 4° C and post-fixed in sym-collidine-buffered osmium tetroxide, stained en bloc with uranyl acetate, and finally dehydrated in a graded series of alcohols and embedded in a propylene oxide-Epon sequence. Enzyme-affinity gold-stained thin sections (see next section) were stained with

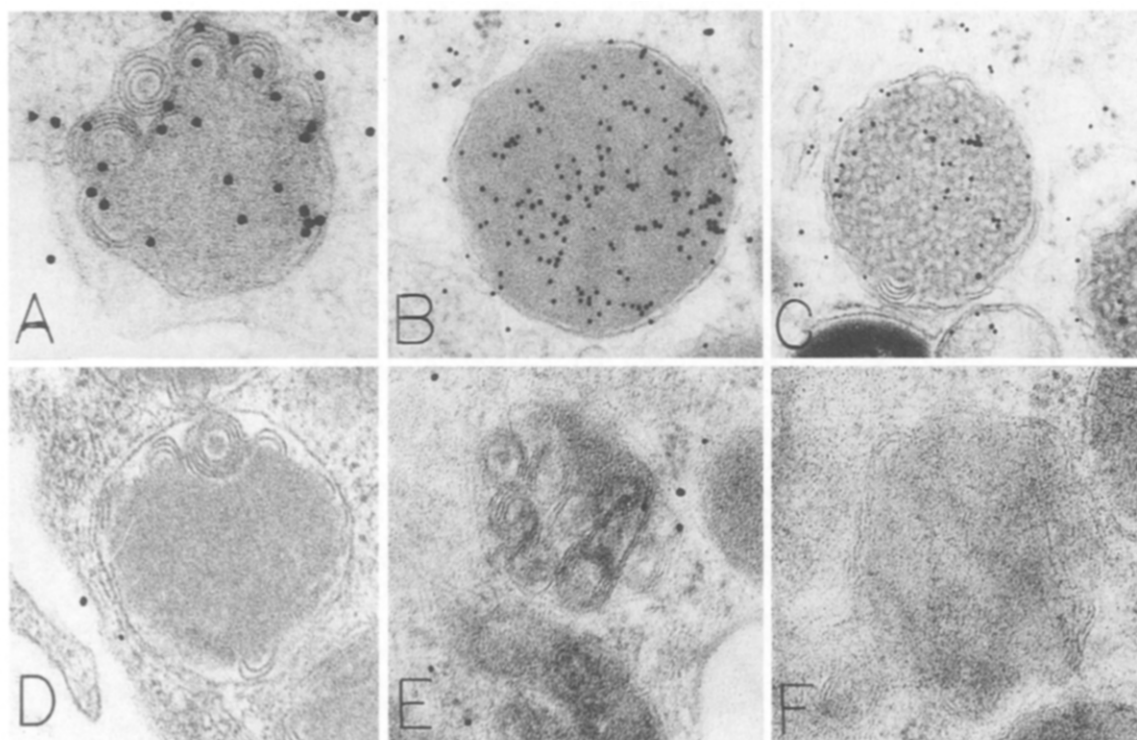


FIG. 2. High magnification micrographs of nonsecretory mast cell granules show DAO-G label indicating histamine in (A) scroll granules, (B) homogeneously dense granules, and (C) particle granules. Scroll granules do not label when DAO-G is absorbed with solid-phase histamine (D) before staining sample, (E) when sample is digested with DAO before staining with DAO-G, or (F) when gold only is used to stain sample. A, Original magnification $\times 67,000$; B, $\times 53,500$; C, $\times 48,000$; D, $\times 54,000$; E, $\times 52,500$; F, $\times 55,000$.

lead citrate and examined in a Philips 300 or 400 electron microscope.

DAO-G enzyme-affinity technique to detect histamine

Thin sections of material embedded in Epon were prepared to demonstrate histamine with the ultrastructural enzyme-affinity method based on DAO-G binding.¹⁰ Briefly, preparation of the DAO-G reagent was done as follows. A colloidal suspension of gold was prepared according to the method of Frens.¹⁷ Four milliliters of an aqueous 1% solution of sodium citrate was added to a boiling aqueous solution of 100 ml 0.01% tetrachloroauric acid and allowed to boil for 5 minutes before being cooled on ice. The pH of the colloidal gold suspension was adjusted to 7 with 0.2 mol/L potassium carbonate. Preparation of the DAO-G complex was done according to the method of Bendayan.¹⁸ Three milligrams of DAO was dissolved in 0.3 ml distilled water and placed in a polycarbonate ultracentrifuge tube with 10 ml of the gold suspension. The mixture was centrifuged at 25,000 rpm for 30 minutes at 4°C in a Beckman ultracentrifuge. The DAO-G complex formed a red sediment that was carefully recovered and resuspended in 3 ml 0.1 mol/L phosphate-buffered saline solution containing 0.02% polyethylene glycol at pH 7.6 (final concentration of DAO 1 mg/ml).

For cytochemical labeling, section-containing grids were inverted and floated on phosphate-buffered saline 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 DAO or absorption of the DAO-G reagent by solid-phase histamine.

RESULTS

Ultrastructural pathologic features of human ileal mucosal mast cells show PMD

PMD, is defined as the progressive release of dense secretory granule contents in the absence of granule-plasma membrane fusion. Partially empty and empty granule containers are retained in the cytoplasm of cells undergoing this form of secretion. Eight of 10 cases examined here revealed PMD of gut mucosal mast cells with use of these ultrastructural criteria for identification. Criteria for cell injury to mast cells were absent.

DAO-G labels histamine in human ileal mucosal mast cells

Mast cell granules were extensively labeled for histamine in cells with a complete complement of electron-dense granules (Fig. 1); cells with a mixture of full and empty granules also contained histamine in electron-dense (full) granules. Lipid bodies (large, round, non-membrane-bound osmiophilic structures), present in smaller numbers than granules in the cytoplasm of ileal mast cells, were not labeled for histamine, thus providing

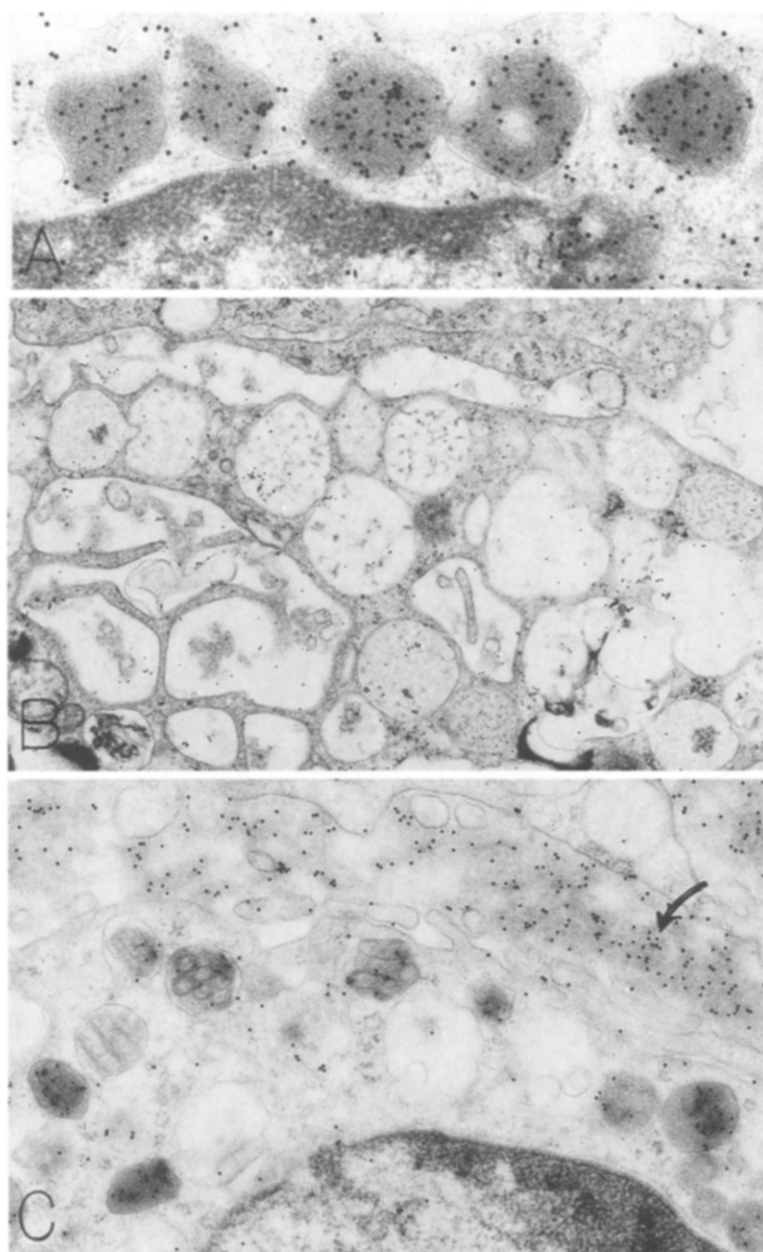


FIG. 3. **A**, Mast cells with electron-dense granules remaining in secretory cells show gold-labeled granules indicating histamine. One granule shows focal, piecemeal loss of dense material and absence of gold label for histamine in this area. **B**, Marked losses of electron-dense contents of partially empty and empty granule containers in mast cells undergoing PMD, show little to no gold label for histamine. **C**, Another mast cell undergoing PMD, shows extensively gold-labeled extracellular electron-dense material indicating bound histamine (arrow) external to plasma membrane. **A**, Original magnification $\times 37,000$; **B**, $\times 19,500$; **C**, $\times 25,000$.

an internal negative control within the same cells. The ultrastructural granule patterns generally seen within human ileal mast cells include scrolls, particulate material, homogeneously dense material, and mixtures of these materials; all granules that illustrated these contents were labeled with DAO-G, indicating the presence of histamine (Fig. 2, *A*, *B*, and *C*). Specificity controls for the DAO-G method showed abrogation of human mast cell

granule histamine label when the DAO-G reagent was absorbed with histamine-agarose beads before use (Fig. 2, *D*) or when samples were digested with DAO (Fig. 2, *E*) before staining with DAO-G. Gold alone also did not label mast cell granules (Fig. 2, *F*). Some mast cell nuclei were also labeled with DAO-G, a finding that we have previously attributed to the presence of nuclear polyamines.¹⁰

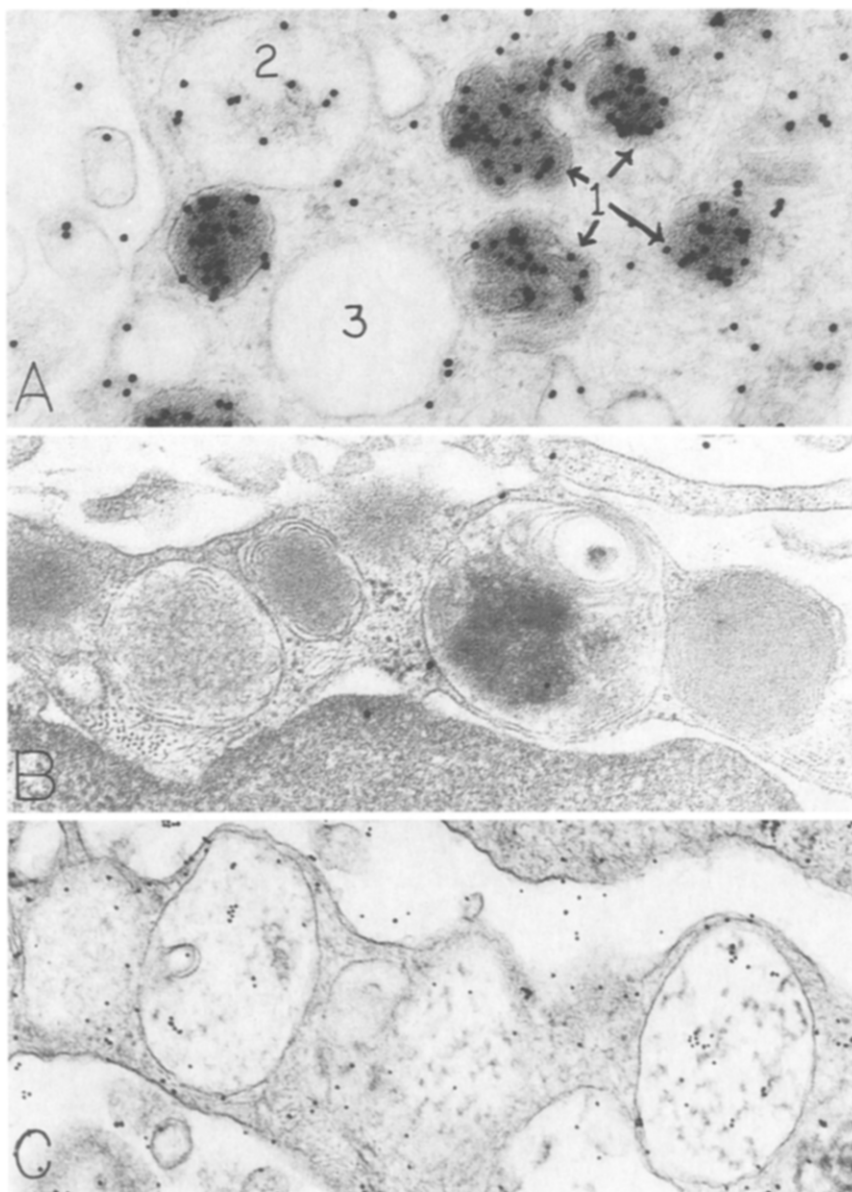


FIG. 4. **A,** At higher magnifications, mast cell granules from cells undergoing PMD show gold label for histamine in electron-dense granules (1), gold label associated with residual electron-dense material in a partially empty granule (2), and no label in an entirely empty granule (3) in a cell undergoing PMD. **B,** Absorption of DAO-G reagent with histamine-agarose before staining abrogates all mast cell granule label. **C,** Residual electron-dense reticular arrays contain small amounts of gold label for histamine in otherwise electron-lucent, empty, histamine-free granules of a mast cell undergoing PMD. **A,** Original magnification $\times 48,000$; **B,** $\times 39,500$; **C,** $\times 42,000$.

Histamine stores are released from human ileal mast cells during PMD in vivo

The empty granule containers in human ileal mast cells undergoing PMD, did not contain gold, indicating loss of enzyme-reactive histamine stores (Figs. 3 and 4). Also, partially empty granule containers retained histamine in the remaining electron-dense components of these structures (Fig. 4, A). Basophils are granulocytes that also contain histamine in their electron-dense secretory granules. Although these granulocytes were rare in the mate-

rial examined, they too underwent PMD, and in such cells the DAO-G label was reduced in the partially empty and completely empty granules. Small, perigranular cytoplasmic vesicles were gold labeled in basophils.

Interlacing bands of interstitial collagen were often increased in areas near degranulating mast cells. These fibrils, as well as amorphous, extracellular, electron-dense matrix material adjacent to secretory mast cells, often displayed heavy labeling with DAO-G, suggesting that released histamine was bound to these structures

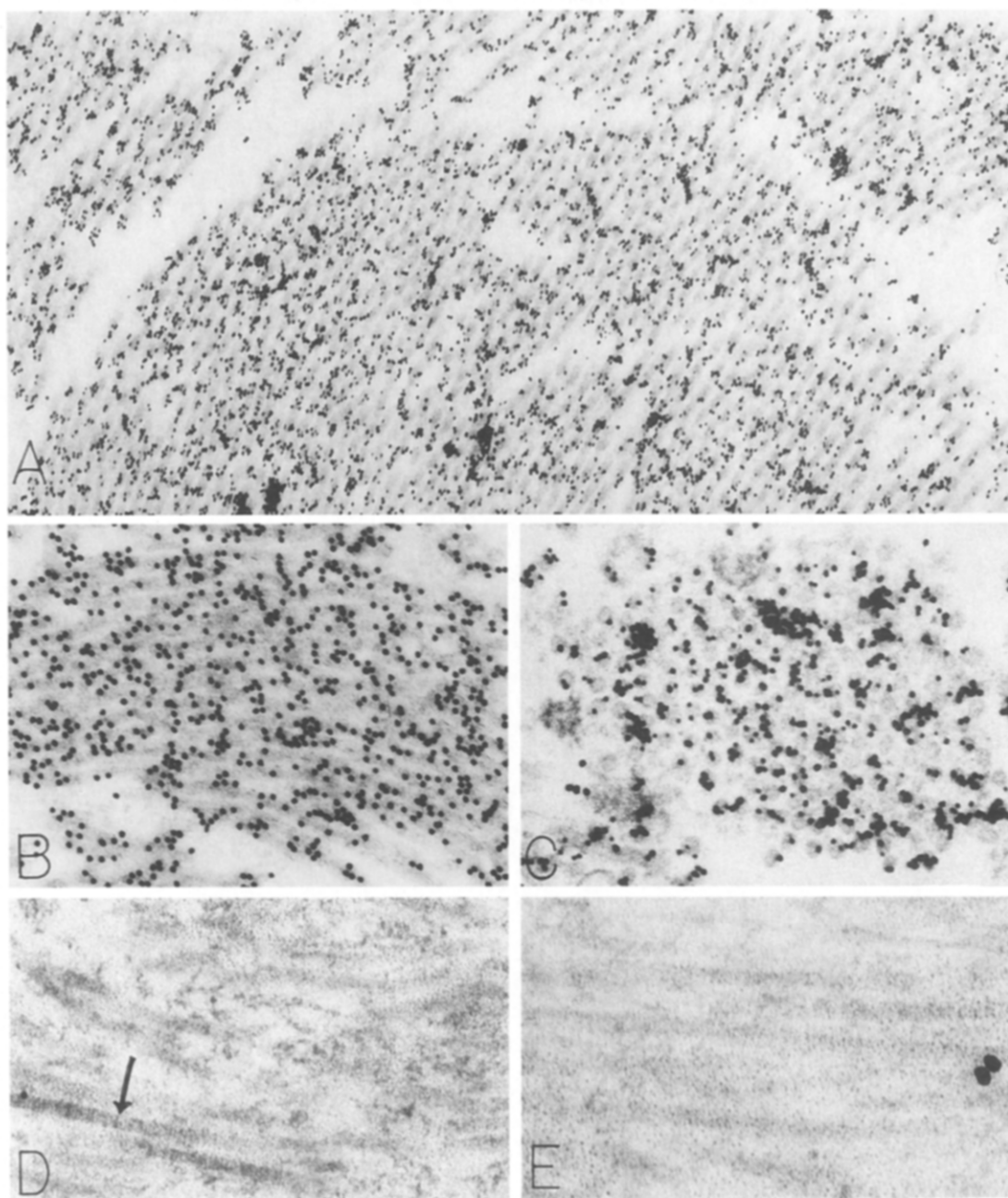


FIG. 5. **A** through **C**, Electron micrographs of collagen-rich extracellular matrix of ileal mucosa (near degranulating mast cells, not shown) show heavily DAO-G-labeled interstitial collagen, indicating bound histamine. Higher magnifications reveal collagen in **(B)** longitudinal section and **(C)** cross-section. Histamine absorption **(D)** and histaminase digestion **(E)** controls are negative. Typical banding pattern of interstitial collagen fibrils (arrow) is well visualized in **D** but is somewhat obscured by overlying dense gold label in **A** through **C**. **A**, Original magnification $\times 24,500$; **B**, $\times 40,000$; **C**, $\times 42,500$; **D**, $\times 63,000$; **E**, $\times 100,500$.

(Figs. 1, *A*; 3, *C*; and 5, *A* through *C*). (The same specificity controls, indicated previously, revealed abrogation of this extracellular label: Fig. 5, *D* and *E*.) Another type of collagenous structure (i.e., basal lamina) lining basilar epithelial, endothelial, and Schwann cell surfaces did not, however, bind DAO-G.

DISCUSSION

The principal findings of this study are as follows: (1) HGMCs have histamine-rich electron-dense granules; (2) histamine is present in electron-dense mast cell granules regardless of their variegated underlying electron-dense substructure; (3) partially and completely empty

granule containers in HGMCs undergoing PMD, in vivo contain little to no enzyme-reactive histamine; (4) enzyme-reactive histamine is bound to interstitial collagen in areas adjacent to secretory mast cells, whereas basal laminae of other cell lineages in the same areas do not bind DAO-G; and (5) histamine absorption and histaminase digestion controls are negative for both electron-dense HGMC granules and interstitial collagen.

Data obtained with the new method used here to detect substructural histamine sites in HGMCs displaying PMD, document the ultrastructural morphology of human mast cell histamine secretion in vivo for the first time, confirming similar observations in the inflammatory eyelid lesions of IL-4 transgenic mice.¹¹ Together these studies clearly demonstrate that the secretory process termed *piecemeal degranulation* is biologically relevant in in vivo HGMCs in inflammatory bowel disease and FP, as well as in mouse skin mast cells expanded in vivo in the eye disease associated with the overexpression of IL-4.¹¹

PMD was first described in human cells with a general degranulation model conceptualized in 1975 in routine ultrastructural studies of human basophils that were infiltrating experimentally elicited skin contact allergy lesions.¹⁹ Before these studies, vacuolization of human metachromatic granulated cells was generally thought to reflect the solubilization of granule materials in aqueous solutions that were used for the preparation of electron microscopic samples, despite the obvious fact that many granules in the same cells were not so depleted (reviewed in Dvorak²⁰). Dissent to this opinion existed,²⁰ with support that included the elegant demonstration that rat mast cell granules were not water soluble, despite days of exposure to water.²¹ The enzyme-affinity gold method that we used to detect histamine in mast cells^{10, 11, 22, 23} and basophils²⁴⁻²⁶ and that we report here and elsewhere¹¹ substantiates secretion of histamine from mast cells in vivo in two species. These studies also correlate this mode of secretion with the ultrastructural development of electron-lucent, empty containers devoid of electron-dense materials and histamine in vivo.

In addition to morphologic studies,³ other studies of mast cell biologic features have implicated these cells in the pathogenesis of inflammatory bowel disease.⁵⁻⁹ Significant findings in these studies include an increased histamine content^{5, 7, 8} and secretion^{5, 8, 9} in the mucosa of patients with CD and UC; jejunal release of prostaglandin E₂ in patients with CD and their first-degree relatives, unrelated to disease activity⁶; and suppression of mast cell secretion by drugs that are effective in the therapy of inflammatory bowel disease.²⁷⁻²⁹ The histamine degradation enzyme, DAO, is generally confined to the small bowel mucosa³⁰ and, in humans, is liberated into the blood after intravenous heparin infusions. Several studies have shown that postheparin plasma DAO levels are markedly reduced in patients with active small bowel CD and are returned toward normal in disease remission.^{31, 32} These values are indicative of small bowel mucosal disease in CD. They may

reflect mucosal damage and repair in this site and certainly may render patients with CD and tissues in CD more susceptible to the biologic effects of endogenously secreted mast cell histamine^{1, 5-9} and exogenously ingested sources of dietary histamine (for example, red wines, spoiled fish).³³⁻³⁵

Slow secretory responses of HGMCs may be a source of preformed proinflammatory mediators, such as histamine and newly generated products of arachidonic acid metabolism, in chronic inflammatory processes like those present in inflammatory bowel disease. Mast cells are also rich sources of cytokines,³⁶ and these products may also be similarly released to tissues by secretion resembling PMD.³⁷ It is now clear that numerous cytokines and growth factors bind to various extracellular matrix materials from which they are available for interactions with cells over time.³⁸ Many cytokines bind to heparin in the extracellular matrix³⁹⁻⁴¹; histamine might also bind to heparin also released from mast cells or to collagen-bound heparin, or to both. The DAO-G technique that we used to detect histamine clearly resulted in binding to the proteoglycan-rich exterior surfaces of interstitial collagen fibrils near secretory mast cells. Moreover, this binding was specific and resisted fixation and processing in a nonmembrane-contained form. Other collagen-rich structures, such as the basal lamina lining endothelial, epithelial, and Schwann cell surfaces in the same sites, did not stain with DAO-G, indicating the lack of histamine bound to these boundary layers, which are composed of type IV collagen.

Another mechanism for binding soluble histamine, which, is slowly secreted from HGMCs undergoing PMD, in inflammatory bowel disease tissues, could be by transglutamination.⁴²⁻⁴⁵ Transglutaminases are calcium ion-dependent enzymes that catalyze an acyl transfer reaction via a protein-protein crosslinkage, resulting in ϵ -(γ -glutamyl)-lysine bonds.⁴⁶ There is evidence implicating increased transglutaminase enzyme activity in murine mast cells activated to secrete histamine.⁴⁷ Transglutaminases are involved in a variety of biological events requiring tissue stabilization.⁴⁸⁻⁵¹ One of these is clot stabilization by circulating factor XIIIa.^{45, 48} Patients with CD have decreased blood levels of factor XIIIa,^{52, 53} a test that has therapeutic implications because this level is most reduced in patients with active disease and, in particular, in patients with refractory fistula formation.⁵³ Immunohistochemical studies colocalized both serum (factor XIIIa) and tissue transglutaminases to the extracellular matrix of damaged tissues in Crohn's disease,⁵² a site uniquely available to aid in the crosslinkage of mast cell-secreted histamine to adjacent extracellular collagen fibrils. Thus a bound source of extracellular histamine, in addition to cytokine, may be of pathogenetic relevance in inflammatory bowel disease.

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