Piecemeal Degranulation of Mast Cells in the Inflammatoty Eyelid Lesions of Interleukin-4 Transgenic Mice. Evidence of Mast Cell Histamine Release In Vivo by Diamine Oxidase-Gold Enzyme-Affinity Ultrastructural Cytochemistry

By Ann M. Dvorak, Robert I. Tepper, Peter F. Weller, Ellen S. Morgan, Patricia Estrella, Rita A. Monahan-Earley, and Stephen J. Galli

We used light and electron microscopy to analyze the eyelid inflammation that develops in transgenic mice that overexpress interleukin-4 (IL-4; Tepper et al., Cell 62:457, 1990). Analysis of alkaline Giemsa-stained plastic sections examined by light microscopy (Dvorak et al., J Exp Med 132:558, 1970), as well as by routine transmission electron microscopy, indicated that the mast cells in the inflammatory eyelid lesions were undergoing piecemeal degranulation, a form of secretion in which the cells' cytoplasmic granules exhibit characteristic morphologic changes that are thought to be associated with the prolonged, vesicle-mediated release of the granules' constituents. Moreover, by using a newly reported enzyme affinity-gold method, which stains histamine based on binding to diamine oxidase-gold (Dvorak et al., J Histochem Cytochem 41:787, 1993), we show that these activated mast cells had released much of their histamine content. The eyelid lesions also exhibited increased numbers of mast cells; interstitial fibrosis, particularly around cutaneous nerves and blood vessels; activated fibroblasts; focal axonal damage; venules with endothelial cells containing numerous vesiculo-vacular organelles; and infiltrates of neutrophils and eosinophils. Our findings illustrate that overexpression of the IL-4 gene in vivo can result in eyelid lesions associated with piecemeal degranulation of mast cells, as well as tissue fibrosis and a variety of other pathologic changes. These results also represent the first direct morphologic evidence for histamine secretion by mast cells in vivo.

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INTERLEUKIN-4 (IL-4) is a multifunctional lymphokine that has a broad range of biologic activities. Originally described as a product of helper T cells, IL-4 is also produced by mast cells. By contrast, the non-T non-B cells that are associated with IL-4 production during some immune responses in mice in vivo have been identified as basophils and their precursors—a cell lineage that exhibits high serum IgE levels, but also develop severe inflammatory disorders, as well as tissue fibrosis and a variety of other pathologic changes. These results also represent the first direct morphologic evidence for histamine secretion by mast cells in vivo.

In the IL-4 transgenic mice correlated directly with the level of the IL-4 transgene expressed in the animals' T lymphocytes. These findings may have relevance to human disease, because IL-4--producing T-cell clones can be derived from the inflamed conjunctiva of patients with vernal conjunctivitis, a basophil-rich inflammatory infiltrate, in greater numbers than from the conjunctiva of control subjects.

Mast cells participate in many allergic or chronic inflammatory disorders, as well as in the host response to neoplasia, in both humans and animals. Our ultrastructural studies indicate that, in settings such as these, mature mast cells can release their granule contents but retain the granule membranes within the cytoplasm; this process has been termed piecemeal degranulation to distinguish this mode of secretion from the explosive extrusion of granules that characterizes anaphylactic degranulation. Although ultrastructural evidence indicates that the process of piecemeal degranulation can result in alterations of granule contents, with a reduction in their electron density, until recently there has been no way to determine whether the cells involved have actually secreted histamine. Histamine is a major mast cell granule component and a potent proinflammatory mediator. Recently, we developed a new postembedding ultrastructural enzyme affinity-gold technology that can localize this mediator at the ultrastructural level. We tested the specificity of the diamine oxidase (DAO)-gold method on cultured human lung mast cells and showed that electron-dense secretory granules contained histamine and that the staining of these granules was abolished by prior digestion with DAO or by filtering the DAO-gold over solid-phase histamine before use. However, we did not assess whether mast cells stimulated to secrete mediators in vivo exhibited alteration of staining with DAO-gold.

In the present study, we evaluated the eyelid lesions that develop in IL-4 transgenic mice using light microscopy of alkaline-Giemsa-stained plastic sections, routine transmission electron microscopy, and enzyme affinity-gold electron microscopy to detect histamine. We found that the tissue...
Fig 1. Light microscopy of alkaline-Giemsa-stained plastic 1-μm sections of eyelids in IL-4 transgenic mice shows piecemeal degranulation of mast cells. In (A), a mast cell with no altered granules is shown to compare with those showing staining alterations of some (B and C) or virtually all (D) of the cytoplasmic granules. The mast cells undergoing piecemeal degranulation (B through D) show many cytoplasmic granules that stain pink, in contrast to the dark blue staining exhibited by the granules in the normal-appearing mast cell in (A). However, the granules with altered staining remain within the cytoplasm. Original magnifications: (A) × 2,200, (B) × 1,500, (C) × 1,700, and (D) × 2,200.

MATERIALS AND METHODS

Transgenic mice. Transgenic mice of the strain designated TG.UD were generated using a fusion gene linking Ig heavy chain enhancer and promoter elements to a murine IL-4 genomic fragment, as previously described.11,27 Age-matched homozygous transgenic and control mice, between 10 and 12 weeks of age, were used for electron microscopic studies.

Routine electron microscopic method. Four IL-4 transgenic and three age-matched control mice were killed by cervical dislocation. Four eyelids exhibiting inflammation of variable severity (from the IL-4 transgenic mice) or three control eyelids (from the normal mice) were excised, immediately trimmed into small blocks (while immersed in a mixture containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 25 mg CaCl₂ in 0.1 mol/L sodium cacodylate buffer, pH 7.4) and then fixed in the same solution at room temperature for 5 hours, as previously reported.28 The specimens were then washed with buffer, osmicated, stained en bloc with uranyl acetate, and dehydrated in a graded series of alcohols before infiltration and embedment in Spurr, a low viscosity embedding medium, as described previously.28,29 Plastic 1-μm sections were prepared and stained with an alkaline-Giemsa stain28,30 before examination in a light microscope. Thin sections (~70 nm) were prepared and lightly stained with lead citrate before examination in an electron microscope.

DAO-gold ultrastructural enzyme-affinity method. The preparation of DAO-gold and the postembedding staining of 70-nm thin sections for electron microscopy has recently been reported.29 The specificity of this method for detecting histamine was demonstrated in a series of experiments using human lung mast cells maintained in vitro.26 Controls for DAO-gold. As previously reported,26 specificity controls included staining with gold chloride only, digestion of the grid with DAO before staining with DAO-gold, and absorption of DAO-gold with solid-phase histamine (or Sepharose only, as a control) before staining the grids.

RESULTS

Light microscopy (LM) of 1-μm alkaline-Giemsa–stained Spurr sections. Plastic-embedded, alkaline-Giemsa–stained 1-μm sections were viewed by LM, and a morphometric approach was used to quantify the number of mast cells per square millimeter of dermis in these specimens (as in Wershil et al31). Large numbers of mast cells (426/mm²) were present in the subepithelial area of the most grossly inflamed of the four eyelids from the IL-4 transgenic mice (Fig 1). By contrast, the values for the other three specimens from the IL-4 transgenic mice, which showed substantially less inflammation, ranged from 118 to 159 mast cells/mm² of dermis. The density of mast cells in the 4 affected eyelids from the E-4 transgenic mice (mean ± SEM, 211 ± 72) was significantly greater than that in the three specimens from the control mice (84 ± 14, P < .03 by the one-tailed Mann-Whitney U test).

Evidence of piecemeal degranulation of mast cells was visible by LM in the diseased eyelids; these changes appeared to be correlated with the amount of clinically apparent inflammation, ie, the most extensive inflammation of the eyelid was associated with the most extensive piecemeal degranulation of mast cells. However, mast cells exhibiting evidence of piecemeal degranulation were observed in all
of the eyelid specimens from the IL-4 transgenic mice. By contrast, no mast cells exhibiting evidence of extensive piecemeal degranulation were observed in any of the specimens from the control mice.

In the LM sections (Fig 1), piecemeal degranulation of mast cells was characterized by partial to virtually complete alteration of the staining characteristics of the cytoplasmic granule contents (Fig 1B through D). The altered granules stained pink in the LM sections (Fig 1B through D), in contrast to the dark blue staining of the unaltered granules (Fig 1A through D). By comparing the cells identified in the LM sections with those observed in the specimen during transmission electron microscopy (TEM) (Fig 2), it was clear that the pink granules remaining within the cytoplasm of the actively degranulating mast cells corresponded to granules that had greatly diminished electron-dense contents by routine TEM, but that retained their membranes and remained in place in the cytoplasm. Moreover, the pink granules observed by LM corresponded to the granules that were devoid of histamine, as determined by enzyme-affinity cytochemistry (see below).

Routine TEM in mast cells. Some mast cells in the inflamed eyelids of IL-4 transgenic mice retained a full complement of electron-dense secretory granules (Fig 2A); others were partially or completely degranulated (Fig 2B and C). Virtually all of the mast cell degranulation observed in these specimens was of the piecemeal degranulation type. We rarely noted the granule-to-granule fusion that is characteristically observed in mast cells exhibiting anaphylactic degranulation. In piecemeal degranulation, granules whose membranes do not exhibit fusion with adjacent granule membranes but that exhibit partial to virtually complete loss of dense granule contents, remain within the cytoplasm of the activated mast cells (Fig 2B and C). Many of these granules contained an altered, moderately dense matrix pattern, and retained a round-to-oval shape, but were often greatly enlarged. Degranulating mast cells exhibited small perigranular vesicles, as well as electron-lucent outpouchings of granule membranes or vesicles attached to granule membranes (Fig 3).

In other respects, the mature mast cells in the eyelid tissues of IL-4 transgenic mice were morphologically like those in control eyelid tissues. That is, the surfaces of these cells had many narrow surface folds, the cells generally were mononuclear and exhibited partially condensed nuclear chromatin, and the cells' ample cytoplasm contained large numbers of cytoplasmic granules but few other organelles (Fig 2A).

Immature mast cells (Figs 4 and 5) were observed in the eyelids of IL-4 transgenic mice, particularly in the specimen from the most inflamed eyelid, but not in those of control mice. Immature mast cells had large, oval nuclei that often displayed large nucleoli. Many of these cells had smaller amounts of cytoplasm than did mature mast cells. Small numbers of electron-dense secretory granules were present in the peripheral cytoplasm. These granules were generally smaller and fewer than those present in cytologically mature mast cells. Immature mast cells displayed many mitochondria.
This electron micrograph of an inflamed eyelid in an IL-4 transgenic mouse demonstrates a large immature mast cell. Note the large, eccentrically located oval nucleus with densely aggregated chromatin and an expanded interchromatin matrix, as well as the ample cytoplasm containing a few small, electron-dense granules in the periphery. Several poorly dense immature granules (closed arrows) and granules containing only small vesicles (open arrows) are located just beyond an expanded Golgi structure (G). The remainder of the cytoplasm is filled with ribosomes, most of which are nonmembrane-bound. A large number of elongated mitochondria are noted, and the cell's surface exhibits narrow surface folds. Original magnification x 13,000.

The immature mast cell population in the IL-4 transgenic mice consisted of mast cells of variable size and with variable numbers of secretory granules (Figs 4 and 5). Other ultrastructural pathologic findings. The eyelid specimens from IL-4 transgenic mice, particularly sections from the grossly most inflamed eyelid, exhibited several other pathologic findings. For example, there was increased amounts of interstitial collagen in the dermis (Figs 5 and 6), particularly around cutaneous nerves (Fig 6) and blood vessels. Numerous elongated fibroblasts with massively dilated cisterns of rough endoplasmic reticulum were distributed among collagen bands, often adjacent to mast cells, in heavily collagenized areas (Figs 5 through 7).

Increased numbers of nerve bundles were also evident in these sections. These nerve bundles were often encased in dense collagen, and in some bundles we noted damaged axons (Fig 6). Postcapillary venules were also more numerous in these samples than in control specimens. They were lined by plump, undamaged endothelial cells that were packed with interconnected vesicles and vacuoles, an endothelial organelle termed the vesiculo-vacuolar organelle (VVO) (Fig 8). Focal collections of these unique organelles, each composed of 20 to 30 individual components, spanned the full thickness of the endothelial cell cytoplasm (Fig 8A). In each thin-section view of postcapillary venules cut in cross-section, as many as 20 individual endothelial cell domains were filled with clusters of VVOs. Despite the morphologic and enzyme affinity-gold evidence (see below) of mast cell secretion of histamine, no interendothelial cell gaps were present (Fig 8A and B). However, clusters of VVOs were focally prominent in parajunctional locations of endothelial cells (Fig 8B). At higher magnifications (Fig 8B and C), individual components of VVOs appeared identical to plasmalemmal vesicles, which were attached to luminal or abluminal endothelial cell surfaces.

The eyelid samples from IL-4 transgenic mice also contained many interstitial neutrophils, some of them in close proximity to mast cells. The neutrophils were well-preserved and fully granulated; some of them had infiltrated the epithelial layer. Eosinophils were also present, although not as
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Fig 5. This low magnification electron micrograph of an inflamed eyelid in an IL-4 transgenic mouse shows a large immature mast cell (M) with small numbers of cytoplasmic granules, a large fibroblast (F) with massively expanded cisterns of rough endoplasmic reticulum (open arrowhead), and two extravasated red blood cells (one of them designated R), all of them adjacent to thick bands of interstitial collagen (solid arrowheads). Original magnification × 7,000.

frequently as neutrophils; eosinophils had not infiltrated the overlying epithelium. Finally, some red blood cells, exhibiting variable loss of their hemoglobin content, were present among bands of collagen (Fig 5).

_Ultrastructural enzyme cytochemistry to detect histamine._ Diamine oxidase-gold stains of the mouse eyelid specimens labeled the electron-dense granules within mast cells, indicating the presence of histamine (Fig 9A through F). However, the altered, swollen granules of mast cells undergoing piecemeal degranulation either did not exhibit DAO-gold labeling or had markedly reduced granule labeling. This finding indicated that these altered granules, which were retained in the cell cytoplasm, contained little or no histamine (Fig 9A, C, and F). Occasionally, perigranular vesicles, or those that appeared attached to the granule membranes, as well as individual fenestrae within the interconnected vesicles of VVOs (Fig 8C), also contained gold particles. Immature granules in mast cells were labeled (Fig 9B), but often were labeled less intensely than were the larger, more condensed mature granules that were present in the same cells (Fig 9B).

Specificity controls for the DAO-gold enzyme affinity ultrastructural method were negative. That is, gold chloride alone did not stain mast cell granules, nor were the granules stained when the DAO-gold reagent was absorbed with solid-phase histamine or when the section-containing grids were digested with DAO before staining with DAO-gold. Passage of DAO-gold over solid-phase Sepharose before staining did not eradicate specific staining of mast cell granules. Eosinophil granules were not labeled for histamine with the DAO-gold technique, providing a good internal negative control (Fig 9G).
DISCUSSION

We show in this study that the eyelid lesions that develop in transgenic mice that overexpress IL-4 contain increased numbers of mast cells and that these mast cells exhibit piecemeal degranulation. We demonstrated the ultrastructural features of mast cell piecemeal degranulation using light and electron microscopic methods that we have previously used to examine piecemeal degranulation of human and animal mast cells in a wide variety of diseases and experimental models. We initially described piecemeal degranulation in basophils that had infiltrated experimentally induced and sequentially biopsied cutaneous contact hypersensitivity lesions in humans. As characterized by LM and electron microscopy, piecemeal degranulation of basophils evolved gradually over 3 days after elicitation of the reaction. Accordingly, we postulated that piecemeal degranulation represented a mechanism for the slow release of basophil or mast cell granule contents, and thus differed from the much more rapid secretion seen in classic IgE-mediated anaphylactic degranulation of mast cells and basophils. Since these early studies, it has become apparent that the most frequently observed ultrastructural evidence of function detected in the mast cells and basophils in biopsies of human disease are those of piecemeal degranulation.

We also studied the inflamed eyelid tissues of the IL-4 transgenic mice, using an enzyme affinity-gold method that we recently developed to localize histamine at the ultrastructural level. This method is based on the affinity of DAO, which binds to its substrate, histamine, even after conjugation to electron-dense gold particles. Moreover, DAO-gold is used as a postembedding stain on routinely and optimally prepared ultrastructural samples, allowing excellent preservation of fine structural anatomy. In the present study, we found that DAO-gold stained the unaltered electron-dense granules of normal mouse mast cells, but staining was markedly reduced or absent in the swollen, altered granules of the mast cells that exhibited evidence of piecemeal degranulation. These findings represent the first ultrastructural evi-
Fig 7. Another electron micrograph of an inflamed eyelid in an IL-4 transgenic mouse shows portions of two granule-containing mast cells (M) adjacent to and focally in contact with an elongated cellular process of a fibroblast (F); this process contains abundant rough endoplasmic reticulum, mitochondria, Golgi structures, and cytoplasmic vesicles. The vesicles (solid arrowheads) are especially prominent near those parts of the fibroblast plasma membrane that are in contact with, or close to, one mast cell. Original magnification × 23,000.

Fig 8. Electron micrographs of routinely prepared specimens (A and B) or of a specimen stained with the diamine oxidase-gold technique to illustrate histamine (C), from an inflamed eyelid of an IL-4 transgenic mouse. VVOs are present in the endothelial cells of postcapillary venules. In (A), a cross-section view of a single postcapillary venule shows portions of five endothelial cells lining the vessel. Two endothelial cell nuclei (N) are visible; individual cells are connected by interendothelial cell junctions (eg, solid arrowheads), none of which show gaps. Focal clusters of vesicles and vacuoles, termed the VVO, span the cytoplasm of the individual endothelial cells that comprise this vessel. In (B), the area bracketed in (A) is shown at higher magnification to illustrate an interendothelial cell junction (solid arrowhead) that is not separated and is guarded by an overlying endothelial cell flap (open arrowhead). VVOs fill the parajunctional cytoplasm adjacent to this junction. Many vesicles comprising these VVOs show fenestrae (eg, arrow). In (C), two gold particles (arrow) are bound to a single fenestra in a single vesicle within the VVO. L, lumen. Original magnifications: (A) × 17,000, (B) × 35,000, and (C) × 63,000.
Fig 9. Electron micrographs of DAO-gold preparations of inflamed eyelids in IL-4 transgenic mice. (A through F) Electron-dense secretory granules in mast cells undergoing piecemeal degranulation are gold-labeled, indicating the presence of histamine in the unaltered cytoplasmic granules of these mast cells. However, there is little or no gold labeling of the swollen granules, which exhibit greatly diminished electron density and an altered granule matrix (A, C, and F). In one mast cell (B), two immature granules show less gold label (arrowheads) than do the mature, electron-dense granules nearby. Eosinophil granules (note their central, electron-dense cores) are not labeled with gold (G). Original magnifications: (A) × 15,000, (B) × 48,000, (C) × 24,000, (D) × 42,000, (E) × 66,000, (F) × 33,000, and (G) × 43,000.
evidence for secretion of histamine from cytoplasmic granules of mast cells in vivo. Moreover, they support our proposed model of piecemeal degranulation, which postulates that this mechanism can account for the slow release of granule contents over time—a process that we propose is accomplished by vesicular transport. Notably, we have also observed piecemeal degranulation of mast cells in the vicinity of IL-4-secreting tumor cells that have been injected into the skin of normal mice (Dvorak et al, unpublished data). This finding supports the conclusion that local overexpression of IL-4 can induce piecemeal degranulation of cutaneous mast cells.

We have not assessed the ultrastructural localization of serotonin in the mast cells of IL-4 transgenic mice. However, it is thought that histamine and serotonin are ionically bound to similar or identical sites in the matrix of secretory granules in murine mast cells. Accordingly, our findings are consistent with the hypothesis that piecemeal degranulation can result in the release of both histamine and serotonin from these cells. On the other hand, we did not find prominent gaps between the endothelial cells of postcapillary venules, such as those seen immediately after the injection of vasactive amines, at sites of inflammation in the IL-4 transgenic mice. This finding could reflect any of several possibilities. Serotonin is a more potent vasodepressor mediator in mice than is histamine. This fact, when taken together with our ultrastructural findings, raises the possibility that piecemeal degranulation of mast cells in the lesions of IL-4 transgenic mice resulted in the preferential release of histamine rather than serotonin. Additional possible explanations of our findings are that the local concentrations of histamine (or serotonin) produced in the vicinity of mast cells undergoing piecemeal degranulation were insufficient to induce the development of interendothelial cell gaps, or that the vascular endothelial cells developed tachyphylaxis to some of the effects of the mast cell-derived mediators.

In addition to piecemeal degranulation of mast cells, the eyelid lesions in the IL-4 transgenic mice also exhibited a number of other ultrastructural features similar to those observed in allergic inflammation or other chronic inflammatory conditions. These findings included many fibroblasts with features of actively synthetic cells, as well as extensive fibrosis, particularly around nerves and blood vessels. Some of these lesions also contained many immature mast cells. Mast cell precursors circulate as agranular mononuclear cells and undergo final maturation and granule synthesis after migration into tissues, a process dependent on stem cell factor (SCF), the ligand for the receptor encoded by c-kit. SCF can be produced by fibroblasts. Moreover, SCF both can induce mouse mast cell activation and mediator release directly and can enhance the mouse mast cell mediator release that is observed in response to IL-4. Taken together, these observations raise the possibility that both the increased numbers of mast cells present in the inflamed eyelid lesions in the IL-4 transgenic mice, as well as the mast cell activation observed in these eyelid lesions, may reflect, at least in part, increased production of SCF by activated fibroblasts at these sites.

Although we have no direct evidence of a change in SCF production by fibroblasts in the eyelid lesions of IL-4 transgenic mice, we did observe greatly increased amounts of interstitial collagen in these lesions. This finding is of particular interest in light of evidence that IL-4 can promote collagen synthesis by fibroblasts in vitro. The eyelid lesions also exhibited infiltrates of neutrophils, as well as smaller numbers of eosinophils, and focal axonal damage. These are not specific findings, as they can be observed in several disorders that might or might not be related to allergic processes, including the gastrointestinal tissues affected by Crohn's disease.

Finally, many of the vascular endothelial cells of venules present at sites of intense inflammation exhibited a complex of interconnected vesicles and vacuoles, termed the VVO. Based on our studies of the augmented vascular permeability induced in the vicinity of growing tumors by the cytokine, vascular permeability factor (VPF), we have proposed that VVOs represent a mechanism by which vessels can exhibit increased vascular permeability in the absence of interendothelial cell gaps, such as those that occur as a result of acute, histamine-induced vascular endothelial cell contraction. We noted extensive VVOs in the endothelial cells of venules in the IL-4 transgenic mouse eyelid lesions; however, interendothelial cell gaps were absent. Although the factors that contributed to the formation of many complex VVOs in the endothelial cells of the IL-4 transgenic mice remain to be defined, the ultrastructural findings suggest that at least some of the tissue swelling observed in this setting may reflect augmented vascular permeability caused by transport through endothelial cell VVOs.

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AM Dvorak, RI Tepper, PF Weller, ES Morgan, P Estrella, RA Monahan-Earley and SJ Galli