Mast cell disruption and other changes in the pericorneal tissue morphology after intraperitoneal injections of a potent histamine liberator (compound 48/80)

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Fluorometric assay for corneal histamine after intravenous injection of compound 48/80 revealed a significant elevation over the level in saline controls. Following intraperitoneal injection of the histamine liberator, compound 48/80, degranulation and disruption of the perilimbal mast cells were noted in whole mount preparations. This was accompanied by accumulation of leukocytes and subsequently round cells in azure eosinate—stained sections of the perilimbal capillary area and morphologic changes in the fibroblasts and endothelial cells. The cellular reaction subsided by 24 hours after the initial injection, although mast cell regeneration continued for at least 14 days. The significance of these findings with regard to the inflammatory reaction and to previous studies using isolated mast cell constituents is discussed.

The demonstration of enhanced wound repair and collagen formation in rat skin associated with prior elevation of histamine-forming capacity by compound 48/80 has opened an attractive new field for investigation of connective tissue repair. A role for the fibroblast in collagen metabolism is well established, but less is known about the tissue mast cell which contains large amounts of histamine and heparin. Treatment with compound 48/80 results in liberation of mast cell granules accompanied by release of histamine. This is followed by phagocytosis of the granules by macrophages, and regeneration of the mast cells. The proposal that the regenerated mast cells are responsible for the elevated histamine-forming capacity has been made. A plausible explanation for the role of histamine in wound healing is offered by Riley who feels that sudden mast cell disruption occurs with liberation of histamine which would enhance local permeability and be followed by the slow release of heparin which would afford a source of mucopolysaccharides for connective tissue metabolism.

The cornea of the eye and the surrounding perilimbal vascular bed affords an accessible area where cellular morphology might be studied. A technique for the study of mast cells in the eye has shown that a rich plexus of mast cells surrounds the cornea but the cornea itself is free of these cells.

The purpose of this investigation was to: (1) determine the histamine content and the effect of compound 48/80 on histamine

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content of normal rat cornea; (2) compare the reaction of the pericorneal mast cell plexus following compound 48/80 injection to the results of previous studies with mesenteric mast cell preparations; and (3) study the effect of mast cell constituents on local cellular morphology without the trauma of injection.

Methods

**Experimental animals.** Male white rats of the Wistar strain from the Naval Medical Field Research Laboratory colony weighing between 300 and 450 grams were used in all phases of the study. All animals were inspected prior to selection for evidence of corneal or intraocular disease and excluded if this were shown.

**Corneal histamine.** Fourteen rats served as controls and 18 rats were injected in the tail vein with 1 mg. per kilogram of compound 48/80 (made up as 1 mg. per milliliter in physiologic saline). The control rats were injected in the tail vein with 1 ml. per kilogram physiologic saline. The rats receiving compound 48/80 became lethargic and showed peripheral cyanosis within 3 minutes which reached a peak within 20 to 30 minutes, at which time they were put to death by cervical fracture without anesthesia. Control rats were also killed at 20 to 30 minutes. Both eyes were immediately removed and the corneas dissected away with particular care not to include scleral tissue. Both corneas from each rat were then weighed and finely ground in a ground-glass homogenizer containing 4 ml. 0.4N perchloric acid and the centrifuged extract assayed for histamine by the orthophthaldehyde fluorometric method of Shore and associates. Due to the possibility of exogenous fluorescent compounds producing an error when small amounts of histamine are assayed, the blank was prepared from the tissue extract.

**Mast cell and tissue structure.** Another series of rats was matched according to weight in groups of ten so that one group could serve as control while a second would receive compound 48/80. The control groups received 1 ml. per kilogram saline intraperitoneally and the experimental group received 1 mg. per kilogram compound 48/80 (made up as 1 mg. per milliliter in saline) intraperitoneally. The rats in group one (test and control animals) were put to death by cervical fracture without anesthesia 1 hour after injection. The animals in group two were killed 6 hours after the first injection, having received a second injection 30 minutes prior to death. The rats in group three were put to death 12 hours after the first injection, having received a second injection at 5½ hours, and groups four, five, and six were killed on the third, seventh, and fourteenth days, respectively, having been repeatedly injected up until the day of death. A single control group was used at the 1 and 6 hour periods but all subsequent groups were individually paired. Two injections were given in the first 6 hour period to insure maximal mast cell disruption with liberation of intracellular components. Immediately after death both eyes were enucleated and one eye was prepared for mast cell examination while the other was used for study of the tissue structure.

The eyes used for mast cell examination were incised at the posterior pole and placed in formalin-alcohol-acetate fixative for at least 24 hours. After removal, the posterior hemisphere was dissected away, and the iris, ciliary body, and retina were gently removed. The tissue was stained in acid toluidine blue and whole mounts were prepared. The technique followed was that of Smelser and Silver.

Mast cell counts were performed with a grid micrometer disk in the microscope ocular calibrated so that ten fields equaled 0.5 sq. mm. Counts were taken from all quadrants in a random fashion, with the criteria that all cells were located within the perilimbal ring, were well distributed throughout the micrometer field, and were intact cells (infra vide).

The eyes for morphologic examination were incised at the posterior pole and the back hemispheres were removed. With the lens in place, they were fixed in Helley's fixative for 24 hours, and washed in tap water 6 to 8 hours. The lens was removed, and the cornea bleached with iodine and thiosulfate, dehydrated through alcohol, cleared in cedar oil, and blocked in paraffin. Cross sections were cut parallel to the anteroposterior axis as near the center of the cornea as possible so that oblique and transverse views of corneal stromal cells would be partially eliminated. Despite the water solubility of mast cell granules, Helley's fixative was used as this method gave the best cytologic detail. The sections were stained with azure eosinate for study of the structure and with toluidine blue for metachromasia. Toluidine blue was used in an attempt to demonstrate any change in sulfated mucopolysaccharide content of the corneal stroma that might reflect diffusion of heparin into the cornea.

A numbering system was established so that the slides could be interpreted using a double-blind experimental design. Statistical analyses were performed with analysis of variance to test for differences in the two corneal histamine groups and the sign test for differences in the mast cell

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*Supplied by Burroughs Wellcome & Co., Inc. (U.S.A.)*
and tissue study. A level of $P = < 0.05$ was accepted as significant.

**Results**

**Corneal histamine.** Table I shows the values of histamine in micrograms per gram of tissue for normal corneal tissue and for corneal tissue during the peak clinical reaction to intravenous injection of compound 48/80. The mean histamine value rises from 6.2 $\mu$g per gram in the normal to 11.6 $\mu$g per gram after compound 48/80 ($P = < 0.01$ [$F = 21.5$ with 1 and 31 degrees of freedom]). All values of histamine are expressed as base. There are no differences in the corneal weights of the control and compound 48/80 groups ($P = > 0.05$ [$F = 1.40$ for 1 and 31 d.f.]).

**Mast cell and tissue structure.** Controls: Mast cells are seen in whole mounts as dense-staining, discrete cells lying in concentric bands immediately outside the cornea. The number of mast cells per 0.5 sq.mm. is summarized in Table II. Only completely intact cells were counted. Those with granules lying around the edges or lacking a well-demarcated nucleus were excluded. Under high magnification, the cells were polygonal in shape with an occasional cell exhibiting granule-containing outward extensions. Heavy granules were closely packed within the cytoplasm and the nucleus could be located by their absence.

The corneoscleral limbus could be easily located on the azure eosinate-stained sections. Mast cells lying close to the vessels and an occasional round cell in the stroma are noted. No differences in any of the metachromatic stains between either controls or compound 48/80 groups could be appreciated at any time.

**Mast cell structure after compound 48/80 injection.** One hour after the initial injection, the perilimbal mast cell count fell from a control of 209.3 to 128.0 cells per 0.5 sq.mm. Many cells exhibited pericellular granules lying in rings of one granule thickness while other cells appeared intact (Fig. 1). By 6 hours, the perilimbal cell count fell to 39.8 cells per 0.5 sq.mm. and is accompanied by widespread mast cell destruction with dispersion of the granules throughout the tissue spaces. A different cell type containing fewer granules of differing sizes and having multiple terminal-branching cytoplasmic extensions is randomly distributed throughout the areas of destruction (Fig. 2). By 12 hours, the number of mast cells rose to 97.0 per 0.5 sq.mm. and these cells now display many forms due to the multiple cytoplasmic extensions (Fig. 3). The intracellular granules again vary considerably in size. Proportionately fewer disrupted cells are noted and the extracellular granularity is not as prominent. By 24 hours, the mast cell count

<table>
<thead>
<tr>
<th>Group</th>
<th>N*</th>
<th>Histamine concentration</th>
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</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>14</td>
<td>6.2 ± 1.99</td>
</tr>
<tr>
<td>48/80</td>
<td>18</td>
<td>11.6 ± 4.0</td>
</tr>
</tbody>
</table>

*SN = number of animals in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after initial injection</th>
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<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td>Saline control</td>
<td>209.3 ± 16.3</td>
</tr>
<tr>
<td>48/80</td>
<td>128.0 ± 17.8</td>
</tr>
</tbody>
</table>

*Significance levels for differences between control and 48/80 groups are based upon the Fisher exact test for the N number of animals in the 48/80 group. No less than nine animals were in any control group. At the 3 day period the $P$-value is calculated from the Fisher formula and probably reflects the small $N$ in the 48/80 group.
Fig. 1. Mast cells in whole mounts of the pericorneal area 1 hour after injection of compound 48/80, showing proximity to blood vessels and pericellular "halo effect" of extruded granules. (Original magnification ×850.)

Fig. 2. Mast cells in whole mounts of the pericorneal area 6 hours after 48/80 injection, showing extensive cellular destruction and the presence of one intact cell with several granule-containing cytoplasmic extensions. (Original magnification ×850.)
Fig. 3. Mast cells in whole mounts of the pericorneal area 12 hours after 48/80 injection, showing intact cells with multiple extensions and pleomorphic granules. (Original magnification ×850.)

is 164.7 per 0.5 sq.mm. Many polygonal cells are seen scattered throughout areas dominated by the cells containing extensions. However, the intracellular granular density of the polygonal cells is less than that of the control cells and the individual granules vary in size. After 3 days of injections, more polygonal cells were seen that were similar to those just described and the count was now 140.5 per 0.5 sq. mm. Very few extracellular granules were found. Seven and 14 days after injection, the mast cell count continued to rise until there was no difference between the saline control and the 48/80 groups on the last day. The mast cells continued to contain fewer granules but became more polygonal in shape.

**Tissue structure after compound 48/80 injection.** In the 1 hour sections, mast cells are noted with "pericellular halos" of granules but vascular congestion and the appearance of acute inflammatory cells in the tissue spaces is particularly prominent. Many polymorphonuclear and eosinophilic leukocytes are seen both within the vessels and in the tissue spaces. In addition, a few cells with round or oval nuclei containing coarse chromatin material and basophilic, pleomorphic cytoplasm without granules are in the perivascular areas. By 6 hours, granules are seen scattered throughout the pericorneal stroma and concentrated within cells having multiple cytoplasmic extensions (Fig. 4). Round cells are now present in large numbers and many contain heavy, basophilic cytoplasmic granules. Blood vessel congestion and granulocytic leukocytes are present. Granules appear in the vicinity of fibroblasts, stromal cells, and endothelial cells but it is not possible to determine whether any are intracellular. Polymorphonuclear and eosinophilic leukocytes are frequently concentrated in areas of mast cell disruption. The nuclei of the fibroblasts, stromal cells, and endothelial cells are rounded and the chromatin lies in scattered clumps giving the entire structure a less dense appearance. The cytoplasm of these cells does not appear to have undergone any change. These changes extend through the thickness of the pericorneal...
Fig. 4. Section through a perlimbal capillary 6 hours after 48/80 injection, showing mast cell destruction (A), leukocyte invasion (B), round cell infiltration (C), and fibroblastic and endothelial cell changes (D). (Original magnification x850.)

tissue but dissipate within a short distance into the cornea. By 12 hours, mast cells smaller than the control mast cells contain granules of varying sizes and exhibit cytoplasmic extensions. The cellular reaction persists and basophilic granules are seen within the cytoplasm of many of the round cells. There is less vascular congestion and the endothelial cells are not different from controls. The fibroblasts vary in size and shape but are not as rounded as in the 6 hour period. At 24 hours, several small mast cells are seen but the general cellular reaction has largely subsided. Vascular congestion was absent but many round cells are still present in the perivascular spaces. Some round cells still contained cytoplasmic basophilic granules. After three, seven, and fourteen days, small mast cells are present but the sections could not be consistently differentiated from controls.

Statistical evaluation. All findings described above are consistent differences from controls in both whole mounts and sections. It was possible to identify all sections as belonging to either control or 48/80 group with an error of only one slide or less through the 24 hour period (sign test, $P < 0.05$).

Discussion

The presence of histamine in the rat cornea is not surprising in view of its ubiquitous nature in the body and particularly high concentration in the rat skin.$^{10}$ Although normal corneal histamine content is considerably higher in the rat than in cattle,$^{27}$ the values are close to the refractory histamine content after maximal release from rat skin by compound 48/80.$^{3,7,15}$ Since there are no mast cells in the nonvascularized cornea, the histamine must either be produced by another type of corneal cell, come from the general circulation, or arise in the local perlimbal mast cells. The rise in corneal histamine content within 30 minutes after injection suggests that corneal cells are not responsible since compound 48/80 releases histamine only from mast cells$^{26}$ and elevation of histamine-forming capacity usually occurs only after repeated injections.$^{7}$ Thus
another source, either systemic or local (perilimbal), may be postulated. The absence of a significant change in corneal weight favors a local source as diffusion from the plasma would be accompanied by edema. Rapid spread of histamine throughout the cornea is plausible in view of the water-soluble diffusion characteristics of the stroma and the presence of hyaluronidase and proteolytic enzymes in the mast cells. A possible source of error that was carefully watched would be inclusion of any portion of perilimbal sclera or conjunctiva.

The appearance of minimal disruptive changes in the mast cells 1 hour after injection of compound 48/80 indicates that significant clinical reactions and cellular infiltration of tissues presumed to be due to release of histamine or other mast cell constituents occur before extensive mast cell disruption.

The tissue sections stained with azure eosinate demonstrated mast cells and granules remarkably well, despite the water solubility of these structures. The vasodilatation and appearance of acute inflammatory cells are consistent with either histamine effect or response to injury.

By 6 hours, maximal mast cell disruption is accompanied by a new cell exhibiting cytoplasmic extensions and containing pleomorphic granules, that may represent one or all of four possibilities. It may represent a delayed stage of dissolution of a mast cell resistant to compound 48/80, since a few normal-appearing mast cells also remain. It may be a mast cell regenerating granules or one undergoing transformation from a local precursor cell. Finally, it may be a macrophage ingesting the dispersed granules.

The disparity in granular size may be an indicator of the resistance of certain mast cell types to compound 48/80, although this has been questioned. If these are previously disrupted, regenerating cells, their appearance at 6 hours is earlier than previously reported and one might expect to observe intact nuclei surrounded by some cytoplasm in the azure eosinate-stained sections of areas where maximal disruption was occurring. The demonstration of morphologic and biochemical differentiation of macrophages and the observation of mast cell formation from mesenchymal, nongranular precursors following local subcutaneous injection of histamine supports the hypothesis of mast cell origin from local precursors. The round cells observed that reached a maximum concentration at 6 and 12 hours appeared to be small lymphocytes which some regard as totipotential cells. The transformation of one cell type into another, different cell morphologically and/or biochemically, either in vivo or in vitro, or both, has been described for the fibroblast, stromal cell, monocyte, macrophage, and lymphocyte. The possible fibroblastic uptake of mast cell granules from the subcutaneous tissue and the similarity at 24 hours of cells in the whole mounts to the "ghost cells" of Riley and West suggest ingestion of granules by macrophages. No conclusions about the nature of these cells can be drawn.

The inflammatory cell reaction is of particular interest. Weimar described the polymorphonuclear reaction of wounded corneas and found that it could be blocked by proteolytic enzyme inhibitors. Subsequently, proteolytic enzymes have been discovered in mast cells. Liberation of endogenous histamine has been implicated in the production of the eosinophilia of magnesium deficiency. The changes in the fibroblasts and cells of the vessel walls have been duplicated by intradermal injections of histamine and heparin in combination but not singly, and were interpreted as a response to the enhanced protein-rich fluid environment. It is possible that all these mechanisms were observed in the experimental groups.

The absence of differences in the tissue metachromatic color reaction between control animals and experimental animals may reflect the high acid mucopolysaccharide content of the cornea that would tend to
obscure subtile changes in tissue heparin content.

Riley's hypothesis of wound repair is supported by the finding of elevated levels of histamine in the cornea before complete mast cell disruption. However, if the cellular reactions and morphologic effects observed in this study are involved in avascular corneal healing, their transient nature would tend to nullify a primary role in this phase of connective tissue metabolism. Smith postulated liberation of "diffusible substances" that would promote corneal vascularization but this was not observed. Finally, the low levels to which the mast cell count rapidly fell and the lack of further destruction indicate that the mast cell constitutes a limited source of substances available for wound repair unless functional changes occur in the remaining intact cells. However, this does not rule out a combined action with other mechanisms of connective tissue metabolism.

This experiment confirms many previous findings associated with mast cell disruption. The elimination of the technical variables of mechanical trauma from injection of substances into an area to be studied histologically and from utilization of tissues (such as rat mesentery) in nonphysiologic preparations affords more evidence that these findings are physiologic effects. No differences in mast cell reaction within the perilimbal plexus and mast cells in other locations seem to exist. The eye thus affords an accessible site for the study of these cells. Although the hypothesis of a role for the mast cell in connective tissue repair is not furthered, this study adds additional evidence for the participation of the mast cell in reaction to injury and inflammatory conditions. Finally, the acute elevation of histamine content after compound 48/80 may be unique to avascular tissue and the role of this substance in corneal physiology offers a challenging area of investigation.

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