

Immunomorphologic studies of mast cell heterogeneity, location, and distribution in the rat conjunctiva

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Mast cells are crucial components of immediate and some delayed-type hypersensitivity reactions. They play a pivotal role in allergic conjunctivitis and other immunoinflammatory disorders of the ocular surface, yet little is known of their distribution and heterogeneity in the conjunctiva of potential animal models, such as the rat. In this study, mast cell types were investigated in histologic sections and corneal-conjunctival-lid whole mounts by using toluidine blue, alcian blue-safranin, and immunohistochemical staining methods (anti-rat mast cell proteinase [RMCP] antibodies). Quantitative analyses were performed on corneal-conjunctival-lid whole mounts by using the optical dissector procedure to obtain the density of mast cells per unit volume in different regions of the conjunctiva. Single and double immunohistochemical analyses revealed that the mast cells in the conjunctiva of the limbus, fornices, and lid margin were strongly RMCP I⁺, suggesting that they were of the connective tissue phenotype. Mast cells containing the mucosal mast cell proteinase RMCP II were not present in the normal conjunctiva. Histochemical analysis revealed that the maturity of the connective tissue mast cells, as assessed by the presence or absence of safranin (heparin)-positive granules in their cytoplasm varied in different regions. In the lid margin 60% to 78% of the mast cells were solely alcian blue-positive, whereas in the fornices 68% to 78% were safranin-positive. In the limbus the predominant type of mast cell was either safranin-positive or contained mixed granules. Mast cell densities were greatest close to the lid margin (10,000 to 12,000 cells/mm³), followed by the limbus (3400 to 4800 cells/mm³) and were rare in the remainder of the conjunctiva (500 to 1000 cells/mm³), with the exception of the region around the nictitating membrane. This study of rat conjunctival mast cells provides essential baseline data for future studies of the role of mast cells in models of allergic conjunctivitis. (J Allergy Clin Immunol 1996;97:1375-86.)

Key words: Mast cell, conjunctiva, eye, rat, immunohistochemistry, optical dissector, heterogeneity, ocular allergy, eyelid, proteinases, mucosal immunology

Mast cells are ubiquitously distributed in connective tissues and are especially numerous at sites of high antigen exposure,¹ such as the dermis² and the mucous membranes of the digestive and respiratory tracts.^{3,4} Cross-linking of the surface-bound IgE by multivalent antigen causes mast cell activa-

tion, resulting in degranulation and secretion of preformed biogenic amines. This response is the principal basis of immediate hypersensitivity reactions. More recently, mast cells have been implicated in a wide variety of non-IgE-mediated immunologic and pathologic processes including responses to parasites and neoplasms, nonspecific chronic inflammatory and fibrotic conditions, angiogenesis, tissue remodeling, and wound healing.^{5,6} In this context it is noteworthy that mast cells are also a rich source of newly synthesized proinflammatory mediators such as prostaglandins, leukotrienes, and cytokines¹ and may be activated or induced to degranulate by other immunologic stimuli such as anaphylatoxins (C5a and C3a), T-cell factors, and products of complement activation.^{7,8} Moreover, nonimmunologic stimuli

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Supported in part by a grant from the Australian Research Council.

Received for publication Sept. 13, 1994; revised July 10, 1995; accepted for publication Aug. 3, 1995.

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0091-6749/96 \$5.00 + 0 1/1/68384

Abbreviations used

AB:	Alcian blue
Ab S:	Alcian blue/safranin
ASF:	Area sampling fraction
CCL:	Corneal-conjunctival-lid
CTMC:	Connective tissue mast cell
MC _T :	Tryptase-positive mast cell
MC _{TC} :	Tryptase- and chymase-positive mast cell
MMC:	Mucosal mast cell
PBS:	Phosphate-buffered saline
RMCP:	Rat mast cell proteinase
SAF:	Safranin

including neuropeptides, venoms, dextran, morphine, heat, cold, and mechanical injury can induce complete or partial degranulation.¹

Mast cells are now known to consist of heterogeneous subpopulations distinguishable by functional and morphologic characteristics.⁹⁻¹¹ In the rat two phenotypes are commonly recognized on the basis of their granular proteinase content: the widely distributed connective tissue mast cell (CTMC) and the mucosal mast cell (MMC), which occurs in the mucosa of the gastrointestinal and respiratory tracts.^{9, 10, 12, 13} Human mast cells also consist of heterogeneous populations differing in neutral proteinase content: one population contains measurable levels of both tryptase and chymase (MC_{TC}), and the second population contains only tryptase (MC_T). The distribution of these mast cells suggests that they are broadly analogous to rat CTMCs and MMCs, respectively.^{4, 14}

The conjunctiva is a unique tissue microenvironment that, like the skin, is exposed directly to light, but because it is a mucous membrane it lacks the protection of a superficial keratinized layer. Furthermore, the conjunctiva has many features in common with the respiratory tract, with which it is continuous, including exposure to airborne environmental allergens. Conjunctival mast cells have been implicated in the pathophysiology of a variety of IgE-mediated allergic conditions affecting the ocular surface, such as vernal keratoconjunctivitis, allergic keratoconjunctivitis (seasonal and nonseasonal forms), and non-IgE-mediated conditions such as giant (contact lens) papillary conjunctivitis.¹⁵ Animal models of ocular allergic responses have largely ignored factors such as heterogeneity and regional variation in mast cell density. In light of the distinct differences in response of the two mast cell types to pharmacologic inhibitors of mast

cell mediator release and the T cell-dependent nature of mucosal mast cells,¹ the initial aim of this study was to investigate mast cell heterogeneity in the rat conjunctiva, for which there is currently no available data. Furthermore, although there are estimates of mast cell density in ocular and adnexal tissues, these have been based on histologic sections.¹⁶ This study used the optical dissector method, which is regarded as the only modern stereological tool capable of estimating particle sizes and number in an unbiased manner.^{17, 18} This quantitative approach was combined with a novel conjunctival whole-mount method to accurately determine the number of mast cells per unit volume in various regions of the rat conjunctiva.

METHODS

Animals

Twelve-week-old female rats (WAG) were obtained from the Animal Resources Centre, Murdoch, Western Australia. Eight rats were used to obtain conjunctival tissue.

Fixation and tissue preparation

After anesthesia was induced with pentobarbital sodium, animals were perfused with phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde in cacodylate buffer. The upper eyelid was marked by a small suture for later orientation. Incisions were made in the skin around the orbital margin, and the globe with attached eyelids was exenterated (Fig. 1, step 1). These were further fixed for 1 hour before dissection. Samples of ears, lymph nodes, and mesentery were removed from some animals to act as positive control tissue. Two methods were used to examine the location, density, and heterogeneity of conjunctival mast cells: conventional histologic sections of paraffin-embedded material and complete cornea-conjunctiva-lid (CCL) whole mounts. In each animal the left eye was processed for whole mounts, and the right eye for histologic studies.

Dissection of CCL complexes for whole-mount preparations

The eyes with lids and intact conjunctival sac were treated as follows. First, the posterior segment of each eye was removed without damaging the conjunctiva (Fig. 1, step 2). The lens was removed, and the anterior segment-lid complex was divided along the horizontal axis at the point of juncture between the upper and lower lids (Fig. 1, step 3). The resulting portions included the cornea, anterior sclera, forniceal conjunctiva, and lid (Plate I, Fig. 2). The iris, ciliary body, and anterior retina were then removed from the deep or internal aspect of each portion (Fig. 1, step 4). The exposed sclera was trimmed close to the limbus. The dissected CCL complexes were placed in 70% ethanol overnight before they were stained and processed. After

staining, the hemi-CCL complexes were further subdivided into three segments before being mounted on glass slides (Fig. 1, step 5; Plate I, Fig. 3a and b).

Histologic procedures

Whole eyes with attached lids were dehydrated and embedded in paraffin wax by using routine methods. Vertical or sagittal sections (8 μ m thick unless otherwise stated) were cut parallel to the pupil-optic nerve axis (Plate I, Fig. 3c). Two sections at different depths were assigned to immunohistochemical studies. For mast cell histochemistry, sections were sampled at five different depths to include representative areas of anterior to posterior conjunctiva. Samples of skin, lymph node, and mesentery were embedded in wax; and sections stained in parallel acted as controls for histochemical and immunohistochemical procedures.

Histochemical staining

After dewaxing and rehydration, sections were stained with either alcian blue/safranin (Ab S) or toluidine blue as previously described.^{13, 19, 20} CCL complexes were stained with either Ab S or toluidine blue by using a modified protocol similar to that described for choroidal whole mounts.^{19, 20} In alcian blue/safranin differential stained preparations, mature CTMCs are strongly safranin-positive (SAF⁺) and appeared red, whereas the intracellular granules of MMCs and immature CTMCs appear sky blue (alcian blue-positive [AB⁺]). In toluidine blue-stained material, mast cells are usually purple or dark purple/blue (i.e., metachromatic), depending on the density of granules.

Quantitation of histochemical appearance of conjunctiva mast cells

As stated above, the alcian blue/safranin staining method may provide information on the state of maturity of CTMCs. Mast cells in the three regions of the conjunctiva (limbus, fornices, and lid margin) were counted in histologic sections from five animals and classified as either red (safranin-positive [SAF⁺]), blue (AB⁺), or mixed (varying proportions of red and blue granules within one cell). Over 800 mast cells at five depths in each specimen were classified with this method.

Immunohistochemical methods

Although Ab S staining provides an indication of phenotype, immunohistochemical analysis of serine proteinases is a more definitive method. In the rat CTMCs contain predominantly rat mast cell proteinase I (RMCP I), and MMCs contain rat mast cell proteinase II (RMCP II). Polyclonal anti-RMCP I and anti-RMCP II antibodies and monoclonal anti-RMCP II antibodies are commercially available for both of these proteinases.²¹ Paraffin sections were stained with cross-absorbed polyclonal antibodies (rabbit anti-RMCP I and sheep anti-RMCP II) at high dilutions (1:5000), as

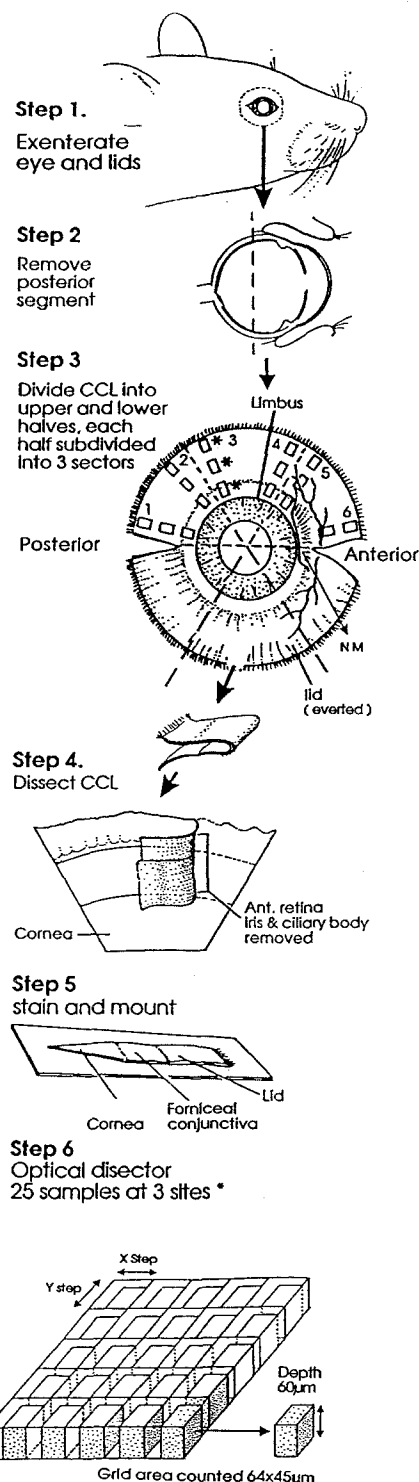


FIG. 1. Diagram summarizing methods used to quantify regional variation in mast cell numbers in the rat conjunctiva. NM, Nictitating membrane.

reported in recent studies from our laboratories.^{13, 19, 20} Nonspecific binding of primary antibodies to mast cells^{22, 23} was prevented by blocking with 0.1 mol/L periodic acid and 0.02% sodium borohydride or 3%

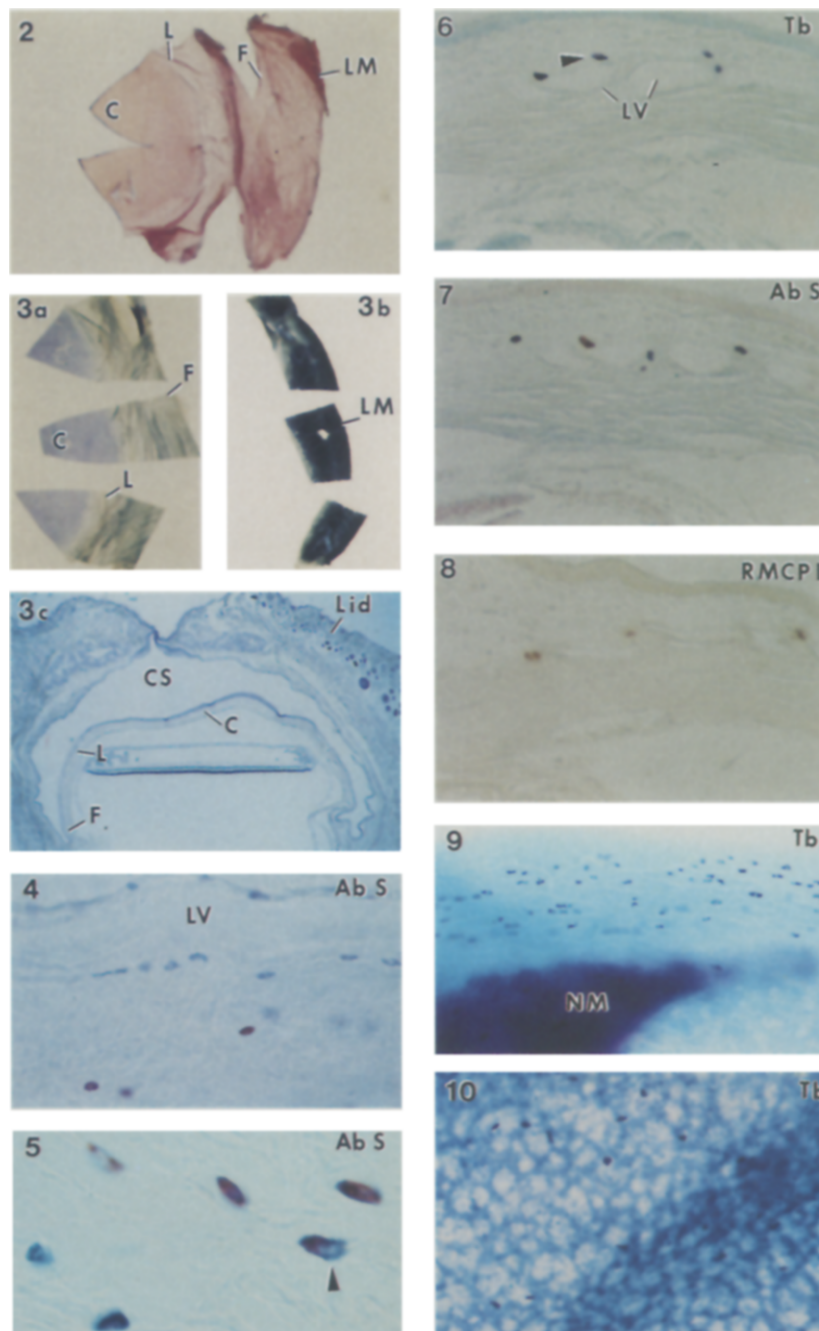


PLATE I. Fig. 2, A CCL complex from a WAG rat stained with Ab S. This intact CCL is from one hemisphere. The cornea (C), limbus (L), loose forniceal conjunctiva (F), and lid margin (LM) can be distinguished. (Magnification $\times 5$.) **Fig. 3a,** The upper CCL from a WAG rat stained with toluidine blue demonstrates the manner in which the corneal (C) and forniceal conjunctival portion (F) were divided into three parts for mounting on slides. **Fig. 3b,** The lid margin (LM) was similarly divided into three portions. L, Limbus. (Magnification $\times 3.4$.) **Fig. 3c,** Vertical (sagittal) histologic section of the anterior segment and upper and lower eyelids from a WAG rat. Note that the conjunctival sac (CS) is bound by the lids, the forniceal conjunctiva (F), the bulbar surface as far as the limbus (L), and the cornea (C). (Leishman's stain; magnification $\times 8$.) **Fig. 4,** "En face" view of the limbus in a rat CCL whole mount. Note that the conjunctival mast cells around the limbal vessels are AB⁺, whereas those further from the vessels are SAF⁺. (Ab S stain; magnification $\times 160$.) **Fig. 5,** Histologic section of perilimbal zone in Ab S-stained preparation. Note that mast cells are AB⁺ (blue only), SAF⁺ (red only), or mixed red and blue (arrowhead). (Magnification $\times 600$.) **Figs. 6 to 8,** Adjacent histologic sections of the rat limbal region. The sections have been stained with various methods to demonstrate mast cells (toluidine blue [Tb], Ab S, and anti-RMCP I antibody). Note that mast cells close to the limbal vessels in these sections are TB⁺, SAF⁺, and RMCP I⁺. (Magnifications: **Figs. 6, 7, and 8** $\times 160$.) **Fig. 9,** Rat CCL whole mount demonstrating the high density of perivascular mast cells in the vicinity of the nictitating membrane (NM). Tb, Toluidine blue stain. (Magnification $\times 110$.) **Fig. 10,** Toluidine blue (Tb)-stained CCL whole mount. The field shows a region of the forniceal conjunctiva in which mast cell density is low. Note the large number of goblet cells (pale ovoid profiles) (Magnification $\times 90$.)

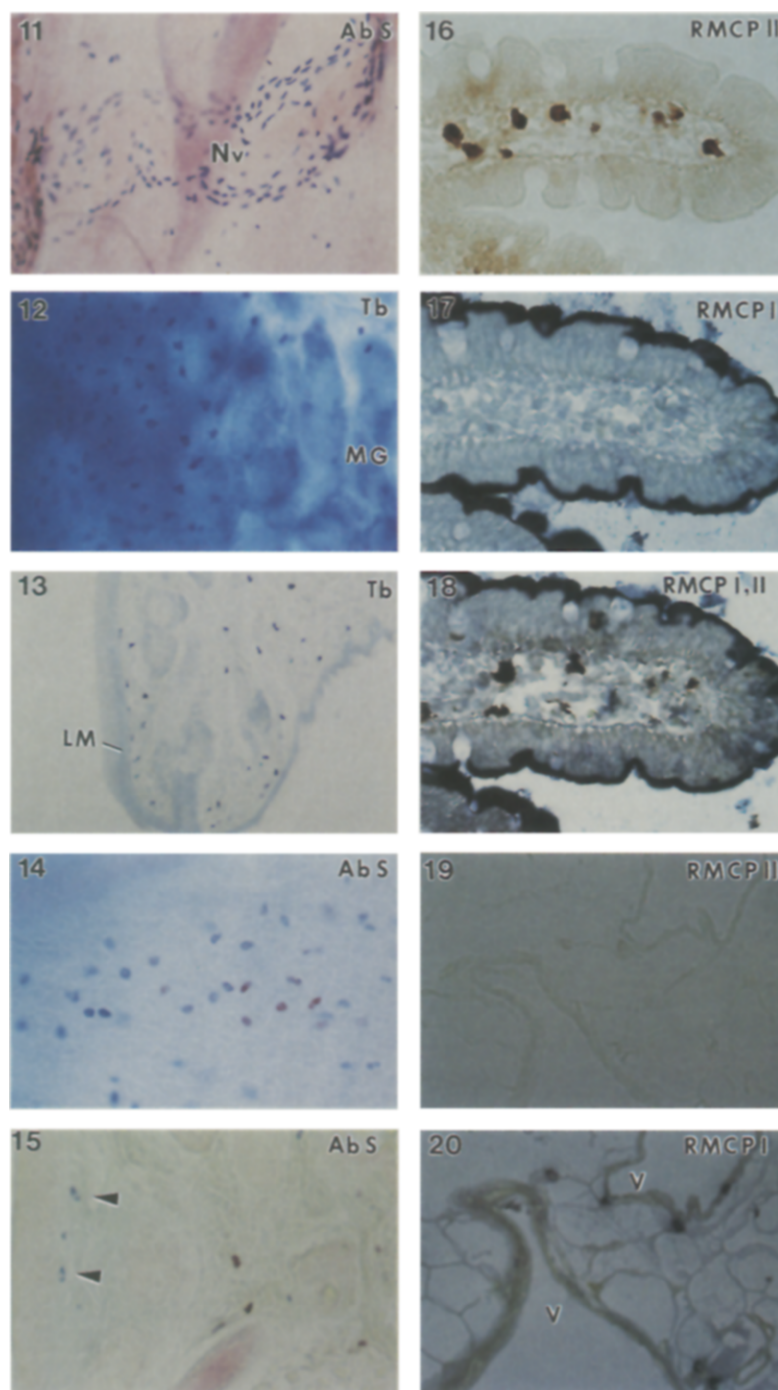


PLATE II. **Fig. 11,** Low-power view of CCL whole mount stained with Ab S. Note the large number of mast cells associated with the conjunctival nerve (Nv). (Magnification $\times 60$.) **Fig. 12,** Low-power view of the rat lid margin stained with toluidine blue (Tb). Note the meibomian glands (MG) lying deep beneath the mast cell-rich conjunctiva. (Magnification $\times 100$.) **Fig. 13,** Low-power view of toluidine blue (Tb)-stained histologic section of rat eyelid. Note the large number of mast cells in the substantia propria beneath the thickened conjunctival epithelium at the lid margin (LM) and in the tarsal connective tissue and dermis. (Magnification $\times 100$.) **Fig. 14,** Whole mount of rat lid margin stained with Ab S. Note that both AB⁺ and SAF⁺ mast cells are present. (Magnification $\times 160$.) **Fig. 15,** High-power view of Ab S-stained sections. Note the AB⁺ mast cells beneath the conjunctival epithelium at the lid margin (arrowheads) and the SAF⁺ mast cells among the meibomian glands and connective tissue deeper in the lid. (Magnification $\times 150$.) **Figs. 16 to 18,** Serial sections of a small intestinal villus from a *Nippostrongylus*-infected rat demonstrating mucosal mast cell hyperplasia. Sections have been stained with anti-RMCP II (**Fig. 16**), RMCP I (**Fig. 17**), and double stained (**Fig. 18**) to demonstrate the specificity of the anti-RMCP II antibody to mucosal mast cells and the lack of RMCP I⁺ CTMCs in this region. (Magnification $\times 340$.) **Figs. 19 and 20,** Adjacent serial sections of fat-bearing region of rat mesentery. Mast cells, distributed among the connective tissue and around blood vessels (V), are of the connective tissue phenotype, namely RMCP II⁻ (**Fig. 19**) and RMCP I⁺ (**Fig. 20**). (Magnification $\times 135$.)

TABLE I. Quantitative evaluation of mast cell histochemical phenotype

	SAF ⁺ (%)	AB ⁺ (%)	Mixed (%)	Mean total (n = 5)
Lower limbus	6 ± 4 (35)	2 ± 1.2 (12)	9 ± 2.3 (53)	17
Upper limbus	4 ± 2.6 (29)	2 ± 1.2 (14)	8 ± 1 (57)	14
Lower fornix	10 ± 3 (78)	0.4 ± 0.5 (3)	2.4 ± 1.6 (19)	12.8
Upper fornix	10 ± 4 (68)	1 ± 1.2 (7)	3.8 ± 2.8 (26)	14.8
Lower eyelid margin	6 ± 5 (11)	32 ± 3.5 (59)	16 ± 13 (30)	54
Upper eyelid margin	3 ± 5 (6)	40 ± 5 (78)	8 ± 4 (16)	51

Data were collected from five sections per eye from a total of five WAG rats. Values are expressed as means ± SD.

H₂O₂ followed by 10% bovine serum albumin in PBS. The primary antibodies were localized with either biotinylated donkey anti-rabbit or donkey anti-sheep horseradish peroxidase directly conjugated secondary antibodies. Positive controls for CTMCs included sections of skin, lymph node, and mesentery. Sections of small intestine prepared from rats infected with *Nippostrongylus brasiliensis*, which display a characteristic MMC hyperplasia, acted as positive controls for this type of mast cell. Negative controls were prepared by substituting PBS for primary antibody. Immunolabeled cells were visualized by using 3'3' diaminobenzidine (brown reaction product). Slides were lightly counterstained with hematoxylin.

Double immunostaining of mast cells

A double immunoperoxidase procedure was used to investigate the extent of coexpression of mast cell proteinases (RMCP I and RMCP II) within the same cell. The procedures were performed on 4 µm thick paraffin sections of conjunctiva and positive control tissues (see Histologic procedures) placed on the same slide. Several series of consecutive slides were assigned to single immunoperoxidase staining (RMCP I alone and RMCP II alone), double staining (both RMCP I and RMCP II), PBS control, or toluidine blue. Double staining was performed in the following sequence: anti-RMCP II, donkey anti-sheep horseradish peroxidase conjugate, anti-RMCP I, donkey anti-rabbit (biotinylated), streptavidin-alkaline phosphatase, alkaline phosphatase substrate (naphthol AS MX and Fast blue), and finally, 3-amino-9-ethyl carbazole. The alkaline phosphatase substrate is visualized as a blue reaction product (RMCP I⁺), whereas the 3-amino-9-ethyl carbazole is visualized as a red reaction product (RMCP II⁺). Double-stained cells, if present, would appear purple.

Quantitation of mast cell numbers

Other authors have estimated the number of mast cells per unit volume within various tissue compartments of the rat eye and adnexa by analysis of cell

profiles in tissue sections.¹⁶ Such methods are now widely believed to be inaccurate and unreliable, and new unbiased stereological tools developed by Sterio²⁴ and modified by Gunderson et al.^{17, 18} are now the accepted and preferred methods of estimating cell numbers in tissues. These methods, including the optical dissector procedure, modified for use in this study, have the advantages of being less time-consuming and more accurate and requiring fewer samples. The optical dissector was applied to CCL whole mounts in this study, thus avoiding the need to cut thick tissue sections, the normal means of sampling used in the dissector method. The principles of the optical dissector method are still relevant to tissue whole mounts.¹⁷

The method used in this study is summarized diagrammatically in steps 3 and 6 in Fig. 1. Toluidine blue-stained CCL complexes were used to quantify mast cells per unit volume. Mast cells were counted in small grids (volume = 64 × 45 × 60 µm) determined according to the method of Gunderson et al.¹⁷ and West et al.¹⁸ on the basis of the average size of the structure to be counted. Twenty-five grids were counted in a 5 × 5 square lattice at preassigned sites on the CCL whole mount, namely the limbus, forniceal conjunctiva, and lid margin (Fig. 1, steps 3 and 6). The fields of vision were systematically selected by the step motor connected to the object stage of the microscope (Olympus microscope fitted with optical dissector motorized stage [Heidenhain, Traunrheut, Germany]). The x and y steps were between 85 and 100 µm in size.

Area sampling fraction (ASF) was calculated as the proportion of the area of the grid of the total x and y steps (Plate I, Fig. 2), namely

$$\text{ASF} = \frac{\text{Area of grid}}{\text{Area of } x \times y}$$

$$\text{Volume} = \text{No. of grids} \times \text{depth} \times (x \times y)$$

A square of grids was counted at each of six regions at all three anatomically distinct sites (limbus, forniceal conjunctiva, and lid margin) for the upper and lower halves (Fig. 1, step 3). This provided a total of

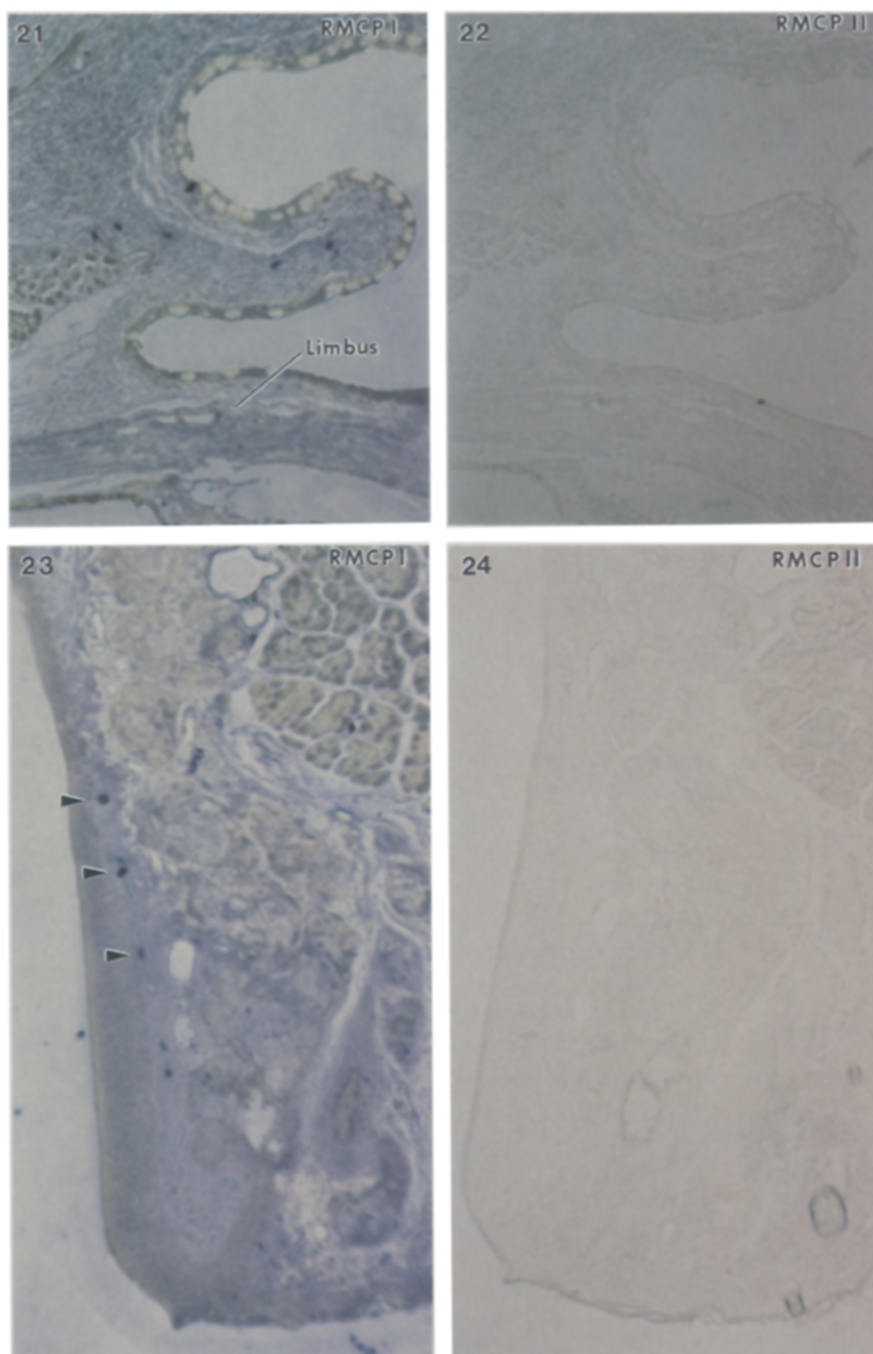


PLATE III. Figs. 21 and 22, Adjacent serial sections of rat limbus and forniceal conjunctiva stained with RMCP I (**Fig. 21**) and RMCP II (**Fig. 22**). Note that there are no RMCP II⁺ mast cells. (Magnification $\times 130$.) **Figs. 23 and 24,** High-power views of adjacent immunostained sections of rat lid margin. Note that the subepithelial mast cells are RMCP I⁺ (*arrowheads* in **Fig. 23**) and RMCP II⁻ (**Fig. 24**). (Magnification $\times 200$.)

150 grids counted for each tissue site in both the upper and lower portions. Within each field of vision or grid, the focal plane was adjusted to the epithelial surface of the conjunctiva. All mast cells that came into focus down to a depth of 60 μm within the area of the grid ($64 \times 45 \mu\text{m}$) with the forbidden line

method¹⁸ were recorded (N). The total number of cells per unit volume is calculated according to the formula:

$$\text{Cells/mm}^3 = \frac{(N/\text{ASF})}{\text{Volume}}$$

TABLE II. Regional variation in mast cell numbers in rat conjunctiva

Segment	Limbus*	Forniceal*	Eyelid margin*
Upper (n = 4)	4,854 ± 539†	1,081 ± 196	10,301 ± 404
Lower (n = 4)	3,410 ± 200	511 ± 160	12,630 ± 598†

Values are expressed as group means (cells/mm³) ± SEM.

**p* < 0.001.

†*p* < 0.05.

This value is calculated for each animal at each anatomic location, and a group mean and standard error were calculated. Statistical analysis was performed with the Student's *t* test.

RESULTS

Mast cell location and phenotype

The following description is based on a combination of observations made on histochemical and immunohistochemical studies (single and double) of paraffin sections and histochemically stained whole mounts.

Limbus (Plate I, Figs 4 to 9). Large numbers of mast cells are located along limbal vessels where they assume an ovoid/ellipsoid, or more rarely, an irregular shape, with their long axis parallel to the vessel, although a few appeared to be wrapped circumferentially around the vessel. Over 50% of these cells contained both AB⁺ and SAF⁺ granules of uniform shape and size (Plate I, Figs. 4, 5, 7) and a third were SAF⁺ (Table I). Cells that contained only AB⁺ granules comprised 12% to 14% of the population (Table I). Mast cells not in the immediate perivascular space were generally more rounded in shape and almost exclusively SAF⁺ (Plate I, Fig. 4). Limbal mast cells also stained strongly with toluidine blue (Plate I, Fig. 6). Single immunohistochemical staining revealed that limbal mast cells were RMCP I⁺ (Plate I, Fig. 8) and RMCP II[−]. The nictitating membrane, which borders the anterior or nasal limbus (Fig. 1), was rich in mast cells (Plate I, Fig. 9).

Forniceal conjunctiva (Plate I, Fig. 10 and Plate II, Fig. 11). For the purposes of this study, the term *forniceal conjunctiva* refers to the zone that includes a large area of conjunctival epithelium and substantia propria, extending from the mast cell-rich limbal zone across the bulbar surface to the fornices from which it is reflected onto the base of the eyelids (Plate I, Fig. 2, 3a, c). The epithelium

is supported by a loose substantia propria, which terminates close to the lid margin where the epithelial thickness increases (Plate I, Fig. 3c). Toluidine blue-stained whole-mount preparations revealed that mast cells were rare in the forniceal conjunctiva (Plate I, Fig. 10); consequently, very few mast cells were included in histologic sections. Histochemical staining and quantitative analysis revealed that the majority of the cells contained SAF⁺ granules. They were either purely SAF⁺ (68% to 78%) or of the mixed AB/SAF⁺ type (19% to 26%) (Table I). In comparison, cells containing only AB⁺ granules were uncommon (3% to 7%). Immunohistochemically these cells contain RMCP I⁺ granules, indicating a CTMC phenotype: cells of the MMC phenotype (RMCP II⁺) were not detected. Occasionally, large nerve bundles traversing the conjunctiva en route to the lids were included in whole mounts. Mast cells were noted in large numbers within the epineurium and perineurium of these nerves (Plate II, Fig. 11).

Lid margin (Plate II, Figs. 12 to 15). Mast cells were extremely numerous in a zone 300 to 500 μm from the lid margin (Plate II, Figs. 12, 13). The population in the substantia propria immediately beneath the conjunctival epithelium consisted of a mixture of AB⁺ and SAF⁺ cells (Fig. 14). Quantitative analysis of histochemically stained sections revealed that a large proportion (59% to 78%) of those mast cells immediately beneath the epithelium were AB⁺ and SAF[−] (Table I). Purely SAF⁺ cells were uncommon (6% to 11%). These observations led us initially to suspect that subepithelial mast cells may be of the MMC phenotype. However, single and double immunostaining revealed that they were RMCP I⁺ and RMCP II[−]. Thus there were no single RMCP II⁺ cells corresponding to MMCs. Large numbers of CTMCs (RMCP I⁺ and SAF⁺) were distributed among the meibomian glands and muscle fibers (orbicularis oculi) of the eyelid distant from the conjunctiva (Plate II, Figs. 13, 15). Mast cells were present in large numbers within the dermis of the lids (Plate II, Fig. 13), which is not an anatomic part of the palpebral conjunctiva.

Double color immunohistochemical analysis of serial sections of control and ocular tissues with anti-RMCP I and anti-RMCP II and double staining with both antibodies were performed to confirm the observations described above. Double immunohistochemical staining of control tissues confirmed the generally accepted pattern of distribution of MMCs (Plate II, Figs. 16 to 18) and CTMCs (Plate II, Figs. 19 and 20). Examination of

stained serial sections of ocular tissues confirmed the presence of strongly RMCP I⁺ mast cells (blue) at the limbus and fornices (Plate III, Fig. 21). Staining of adjacent sections failed to reveal any RMCP II⁺ mast cells (red) (Plate III, Fig. 22). In the lid margin the subepithelial mast cells were strongly RMCP I⁺ (blue) (Plate III, Fig. 23) and RMCP II⁻ (Plate III, Fig. 24). Thus mast cells corresponding to the MMC phenotype were not present in the lid margin.

In summary, qualitative observations of histochemically and immunohistochemically stained preparations have revealed that in the rat mast cells are extremely numerous in the conjunctiva around perilimbal vessels and close to the lid margin. They are less frequent in the intervening conjunctiva, except for a small zone around the nictitating membrane. All rat conjunctival mast cells were of the CTMC phenotype (RMCP I⁺); however, the state of maturation of their granules, as detected by the Ab S histochemical technique, varied considerably. The data suggest that a greater proportion of mast cells in the lid margin were immature compared with those in the forniceal and limbal regions.

Advantages of CCL whole-mount preparations

The whole-mount method developed in this study allowed a much greater appreciation of mast cell numbers and pattern of distribution over large areas of the conjunctiva when compared with conventional sections. Although Ab S-stained whole mounts satisfactorily demonstrated mast cells of various phenotypes and provided important information on the chondroitin sulfate or heparin content of the granules, toluidine blue-stained whole mounts were preferable for quantitative analyses because of the highly contrasting nature of the intensely metachromatically stained mast cells. CCL whole mounts also facilitated investigations of regional variation in mast cell numbers because they allowed quantitation of large and complete areas of conjunctiva, unlike histologic sections.

Quantitative studies of mast cell numbers in the rat conjunctiva

The optical dissector method was used to quantify mast cell numbers in three zones of conjunctiva in 12-week-old WAG rats, namely the limbal conjunctiva, forniceal conjunctiva, and palpebral conjunctiva (within 500 μ m of the lid margin) in both upper and lower segments. The results are presented in Table II.

Quantitative evaluation of eight rats revealed statistically significant differences ($p < 0.001$) between the density of mast cells in the three zones of the conjunctiva; the lid margin had the greatest density, and the forniceal conjunctiva was the least densely populated (Table II). In addition, there were significant differences between the upper and lower conjunctival segments at the limbus and eyelid margin; that is, there are more mast cells in the upper than in the lower limbus, yet at the eyelid margin there are fewer in the upper than in the lower eyelid ($p < 0.05$).

DISCUSSION

In recent years it has become increasingly evident that mast cells play a greater part in immune responses than their classically assigned role in immediate hypersensitivity responses. Thus conjunctival mast cells may play a role in allergic and nonallergic conditions affecting the ocular surface. It was therefore believed to be necessary to undertake this study of mast cell heterogeneity, location, and distribution in the conjunctiva of the rat, a frequently used model of ocular disease.

Accurate phenotyping of mast cells requires a combination of appropriate histochemical and immunohistochemical investigations on suitably fixed tissue.^{3, 9, 25} Histochemical criteria alone are not definitive phenotypic markers, as evident by the Ab S technique in which mature CTMCs stain red because of their high heparin content; however, cells staining blue because of the high granule content of sulfated glycosaminoglycans may be either immature CTMCs or MMCs.^{3, 9, 25} Despite this limitation, several recent studies have mistakenly ascribed cells to the MMC phenotype on the basis of purely histochemical criteria.^{26, 27} Anti-RMCP I and anti-RMCP II antibodies are valuable phenotypic markers of rat mast cell subtypes: CTMCs predominantly contain RMCP I, whereas MMCs are rich in RMCP II²¹; however, a small subpopulation of double-positive cells have been described in a variety of rat tissues.^{28, 29} Analysis of the proteinase content of mast cells in human tissues has led some authors to conclude that it may be more prudent to consider MC_T (equivalent of rat MMC) as cells rich in tryptase and relatively deficient in chymase and the MC_{TC} (equivalent of rat CTMC) to be rich in both proteases.³⁰ Our combined histochemical and immunohistochemical observations show that mast cells of the CTMC phenotype (RMCP I⁺) were widely distributed in the substantia propria of the conjunctiva. Mast cells that were initially suspected to be MMCs on

the basis of either their histochemical characteristics (AB⁺) and/or location around limbal vessels and directly beneath the epithelium close to the lid margin were shown by analysis of single and double immunohistochemical staining to be strongly RMCP I⁺ but RMCP II⁻. No intraepithelial MMCs, similar to those described in other mucosal surfaces,^{31, 32} were identified in the normal rat conjunctiva, a finding in common with normal human conjunctiva.^{33, 34} Our observations support previous studies of mast cell phenotype in the rat conjunctiva, which have inferred a connective tissue phenotype on the basis of their sensitivity to compound 48/80.³⁵ In a study of guinea pig eyelid, Stock et al.³⁶ concluded that what initially appeared to be MMCs were in fact eosinophils or immature CTMCs. Ultrastructural studies of normal human limbal mast cells demonstrated that the majority were morphologically identical to the CTMC type (MC_{TC}), whereas only a minor subpopulation corresponded to the MMC phenotype (MC_T).³⁷ These observations have recently gained support from immunophenotypic studies of human palpebral biopsy specimens, which demonstrated that 97% of mast cells were MC_{TC}.³³

The first reports of variations in mast cell phenotype in pathologic conditions affecting the conjunctiva are emerging and suggest a shift in mast cell phenotype in some diseases. For example, in cicatricial pemphigoid, a shift to the CTMC phenotype²⁶ has been described, and in the case of vernal keratoconjunctivitis, a shift to an MMC phenotype.³³ It is possible that a shift to an MMC phenotype occurs in rodent models of ocular allergy, although this has yet to be investigated.

The perivascular location of limbal mast cells, best visualized in flat mounts, was briefly reported many years ago by Smelser and Silver.³⁹ This location suggests a protective role in local inflammatory responses, that is, altering vasopermeability, blood flow (through vasodilation), and enhancing leukocyte extravasation to sites of local antigen contact or trauma. This study has revealed unexpectedly low mast cell density in a large zone extending from the limbus to the lid margin. Whether this is peculiar to rodents is not clear, because there have been few investigations of the regional variation in conjunctival mast cells in other species. Localized collections of lymphoid tissue or conjunctival-associated lymphoid tissue, a feature of the conjunctiva in other species including human beings,⁴⁰ were not identified in the rat.

In this investigation we describe a novel conjunctival whole-mount technique that enables

“plan” or “en face” views of mast cells over large areas of tissue. The regional variations in mast cell density were quantified in whole mounts by using the new generation of stereological tools for counting and measuring arbitrary particles in three-dimensional space.^{17, 18, 24} This method does not make assumptions about size, shape, and orientation of the particles to be measured, thus providing the only true unbiased estimates. This approach provides the first realistic estimate of mast cell density per unit volume in various regions of the rat conjunctiva. No attempt has been made to estimate total mast cell number in the conjunctiva. This would require the further use of the area sampling fraction method to estimate the volume of the three zones of the entire conjunctiva. Estimates of total mast cell number would be preferable for future experimental studies of allergic conjunctivitis because edema, which accompanies the allergic response, would probably alter the normal depth of the conjunctival substantia propria, thereby affecting estimates of density per unit volume. Appropriate unbiased sampling methods for estimating total numbers in each zone of the conjunctiva, which take into consideration these factors and the extreme variations in density discovered in this study, are currently being developed for future experimental studies.

Our observations support the broad findings of Allansmith et al.¹⁶ who, using nonstereological methods, revealed mast cell densities in the limbus (~2000 cells/mm³) and forniceal conjunctiva (<1000 cells/mm³) similar to those of this study; however, these authors greatly underestimated the numbers in the lid margin at around 1000 cells/mm³. The density at the latter site was found in our study to be between 10,000 and 13,000 cells/mm³, a value close to the estimated 10,000 mast cells/mm³ in the skin² and surprisingly close to the estimates of $11,054 \pm 6327$ cells/mm³ in human conjunctiva.³³ Explanations of regional variations in conjunctival mast cell density must take into consideration interactions between environmental antigens, the ocular surface, and the lacrimal apparatus. After topical exposure to radiolabeled antigen, there is an accumulation of antigen in the lids of rats, probably caused by the sweeping action of the lid margin over the ocular surface during blinking.⁴¹ This may correlate with the greater mast cell density at this site. Interestingly, in the rat we found a 20% higher density of mast cells in the upper tarsal conjunctiva, although rats appear to close both eyelids almost equally during blinking, unlike human beings in whom the lower lid is only raised significantly during

forced blinking. The high proportion of immature CTMCs in close proximity to the basal aspect of conjunctival epithelium at the lid margin may reflect the high antigen exposure and greater rate of degranulation at this site compared with other regions of the conjunctiva. This immature status of these cells manifests itself as an absence of heparin (SAF⁺) granules within their cytoplasm.

Limbal and conjunctival mast cells seem particularly susceptible to nonimmune stimuli because mast cell degranulation induced by mechanical stimuli, such as eye rubbing, can initiate neutrophil and macrophage infiltration of the substantia propria.⁴² The cascade of immunoinflammatory mediators secreted by degranulating mast cells most likely plays a role in conditions in which mechanical irritation is long-lasting and frequently repeated, such as contact lens wear. Indeed, contact lens papillary conjunctivitis is characterized by infiltrates of lymphocytes, macrophages, eosinophils, and neutrophils.⁴³ The link between UV-B irradiation, mast cells, and diseases of the ocular surface remains unexplored. This is despite the fact that conjunctival mast cells lie directly beneath a thin nonpigmented mucosal membrane, a location unparalleled by any other mast cell population in the body. There are published reports of UV-B-induced mediator release from cutaneous mast cells.⁴⁴

The presence of mast cells within the epineurial and perineurial sheaths of conjunctival nerves is a significant observation in light of a variety of convincing evidence that morphologic and functional communication exists between mast cells and the nervous system.⁴⁵⁻⁴⁷ For example, IgE-mediated responses are reduced (23% to 29%) in denervated mouse limbs,⁴⁸ and there is evidence that higher centers in the central nervous system can regulate mast cell secretion.⁴⁹ Our observations are the first to reveal a mast cell-nervous system association in the conjunctiva. Substance P, calcitonin-gene-related peptide, vasoactive intestinal peptide, and neuropeptide Y-immunoreactive fibers have recently been found to be closely associated with mast cells in the lacrimal gland.⁵⁰ Studies colocalizing nerve fiber subtypes and mast cells in the conjunctiva have yet to be performed.

In conclusion, our histochemical and immunohistochemical studies have permitted the accurate phenotypic analysis of mast cell subpopulations in the rat conjunctiva. Furthermore, by using a novel combination of modern stereological methods and tissue whole mounts, we provide the first unbiased

estimates of mast cell density per unit volume in various regions of the conjunctiva. This investigation has provided baseline data for future studies of strain-specific variations and ontogeny of mast cells, and moreover, will form the foundation for further research into the role of mast cells in models of ocular allergy.

We thank Julie Crewe for valuable assistance in many phases of this project. We also thank Dr. Graham Mayrhofer, for kindly providing tissues from *Nippostrongylus*-infected rats, and Dr. H. J. G. Gunderson, for valuable discussions on the methodology.

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