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A new technique for staining mast cells using ferroin

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Abstract

We describe here a new method for specific staining of mast cells using ferroin. Different hamster tissues were fixed in 4% formalin and processed for paraffin embedding. Sections were stained with hematoxylin followed by ferroin acidified with 2.5 N sulfuric acid to pH 4.0. Mast cells stained an intense orange color that contrasted markedly with bluish violet nuclei. High contrast was also observed when ferroin colored sections were counterstained with light green instead of hematoxylin. To evaluate the specificity of the stain, hamster cheek pouch sections were stained with toluidine blue, alcian blue-safranin O, and ferroin. Quantitative evaluation of mast cells stained with the three techniques showed no statistical difference. The simplicity and selectivity of this method is sufficient for image analysis of mast cells.

Key words: ferroin, heteroglycans, ligand dyes, mast cells

Mast cells are present in connective tissue, particularly in the vicinity of blood and lymph vessels. These cells are characterized by the presence of metachromatic basophilic granules in their cytoplasm. These granules contain several chemical mediators including histamine, prostaglandins, heparin, cytokines, proteases, and acid hydrolases (Holmgren and Wilander 1937, Lagunoff et al. 1964, Kida et al. 1986, Plaut et al. 1989, Wodnar-Filipowicz et al. 1989, Gordon and Galli 1990).

The type and quantity of chemical mediators stored and/or synthesized in the granules of mast cells allow these cells to be involved in a wide variety of biological processes, e.g., inflammatory reactions, degradation of the extracellular matrix, processes of angiogenesis, and hypersensitivity (Wershil et al. 1987, Burd et al. 1989, Gordon and Galli 1990, Bissonnette et al. 1993). Mast cells are a heterogeneous population in terms of functional, morphological and histochemical features (El Sayed and Dyson 1993). Within the context of the functional status of mast cells, associated degranulation capacity and alterations in the biosynthesis of the content of the granules leads in turn to changes in the results of histochemical staining (Bissonnette et al. 1993).

Paul Ehrlich (1879) was the first to describe these cells (*Mastzellen*). Since then, many staining methods have been developed to study these cells (Baker 1958, Thompson 1966, Luna 1968, Lillie and Fullmer 1976). The metachromatic methods employ solutions of thiazine dyes at an acid pH, such as toluidine blue O, methylene blue (Coolidge and Howard 1979, Graham et al. 1994) and Giemsa solution (Gaffney 1983). Other techniques employ copper-phthalocyanines such as alcian blue and astra blue (Scott and Dorling 1965, Miyata and Takaya 1980), staining with neutral red (Allen 1960), and combinations of alcian blue and safranin O (Combs et al. 1965, Enërback 1966a,b; Mayrhofer 1980, El Sayed and Dyson 1993). Furthermore, the use of dyes that chelate cationic metals (metallochromes) including iron hematoxylin, gallocyanine-chrome alum, iron-eriochrome cyanine R has been reported (Prentø 2001, Horobin 2002).

We have observed that the ferroin stains mast cells bright orange with great specificity and allows the use of different nuclear and cytoplasmic dyes as counterstains. These are assets for evaluation by image analysis. The aim of the present study was to develop and evaluate this staining method.

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Materials and methods

Samples

We employed tissue from five 150–200 g six-week-old golden hamsters. We took samples of the cheek pouch, lung, intestine, esophagus, skin, ear, liver and kidney. Much of the work was carried out on the cheek pouch, because it exhibits numerous mast cells suitable for quantitative evaluation. The tissues were obtained from untreated control animals from another study performed in our laboratory (Kreimann et al. 2001). The *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals* (1985) were observed.

The samples were fixed for 24 h in 4% formaldehyde-PBS solution, and routinely processed for embedding in paraffin. For quantitative purposes, serial 3 μm sections were obtained from the cheek pouch samples. One section from each of the five animals was mounted on a single slide to guarantee that all the samples to be analyzed were subjected to the same staining conditions.

The reagents employed were: toluidine blue O (CI. 52040; Merck, Darmstadt, Germany), alcian blue 8GX (CI 74240; Mallinckrodt, St. Louis, MO), safranin O (CI 50240; The British Drug Houses Ltd., London) and ferroin (Ferroin-Lösung, cat. no. 46270; Fluka Chemika. Switzerland).

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Staining procedures

Three staining procedures were employed.

Toluidine blue O

The sections were dewaxed and hydrated to distilled water, stained for 2 min in 0.2% toluidine blue in acetate buffer (pH 4.2) and washed in the same buffer. They were placed in 4% aqueous ammonium molybdate solution for 10 min and washed with distilled water (García del Moral 1993). The sections then were dehydrated, cleared and mounted in Canada balsam.

Alcian blue-safranin O

The hydrated sections were stained with 0.1% alcian blue in 0.7 N HCl for 30 min, washed with 0.7 N HCl, and stained with 0.5% safranin O in 0.125 N HCl for 2 min (Mayrhofer 1980). The sections then were rapidly washed in distilled water, dehydrated, cleared and mounted as above.

Ferroin

The sections were stained with Harris′ hematoxylin for 10 sec and exposed to ammonia water for 30 sec to enhance the blue color. They were washed in water acidified with 2.5 N H_2SO_4 to pH 4.0, and stained in ferroin acidified with 2.5 N H_2SO_4 to pH 4.0 a for 15, 30, 45 and 60 min. The sections were washed once again in water acidified with 2.5 N H_2SO_4 to pH 4.0 and mounted as above. Additional sets of sections were run simultaneously employing pure ferroin at pH 5.0 and 6.0, and ferroin diluted to 50% and 10% in water, both at pH 4.0. Adjacent sections were counterstained with a 0.5% solution of light green (CI 42095; Merck, Darmstadt, Germany) after staining with ferroin and omitting nuclear staining with hematoxylin.

Quantitative evaluation

We counted stained mast cells per tissue area using a micrometric eyepiece, and expressed densities as mast cells/mm^2. The 0 mark of the scale was superimposed on the epithelial basement membrane, and the nucleated mast cells were counted in a 25 μm band of connective tissue underlying the basement membrane. The final magnification was × 500. We counted 111 adjacent fields in each section, i.e., a total area of 0.888 mm^2. The data are expressed as mean ± SEM.

Results

The sections stained with the pure ferroin at pH 4.0 for 30 min exhibited bright orange selective staining of granules in the mast cells of all organs. High contrast was obtained when either Harris′ hematoxylin or light green was used as counterstain (Fig. 1). The solutions of ferroin diluted to 50 and 10% stained mast cells faintly. A similar faint staining reaction was obtained by staining with undiluted ferroin for 15 min. Staining was stronger for staining times exceeding 30 min; however, these staining conditions resulted in faint, orange background staining of tissue components including cartilage, hair, and keratinized epithelia, and a reduction in the intensity of the violet hematoxylin staining of nuclei. The use of pure or diluted ferroin at pH values above 4.0 produced nonspecific strong background staining of all the tissues that interfered with counterstaining.

Quantitative evaluation of mast cells was performed on three sets of slides stained with each of the three staining methods under study (Fig. 2). The mean density values were: 10.41 ± 0.96 for ferroin stained sections, 10.97 ± 1.06 for toluidine

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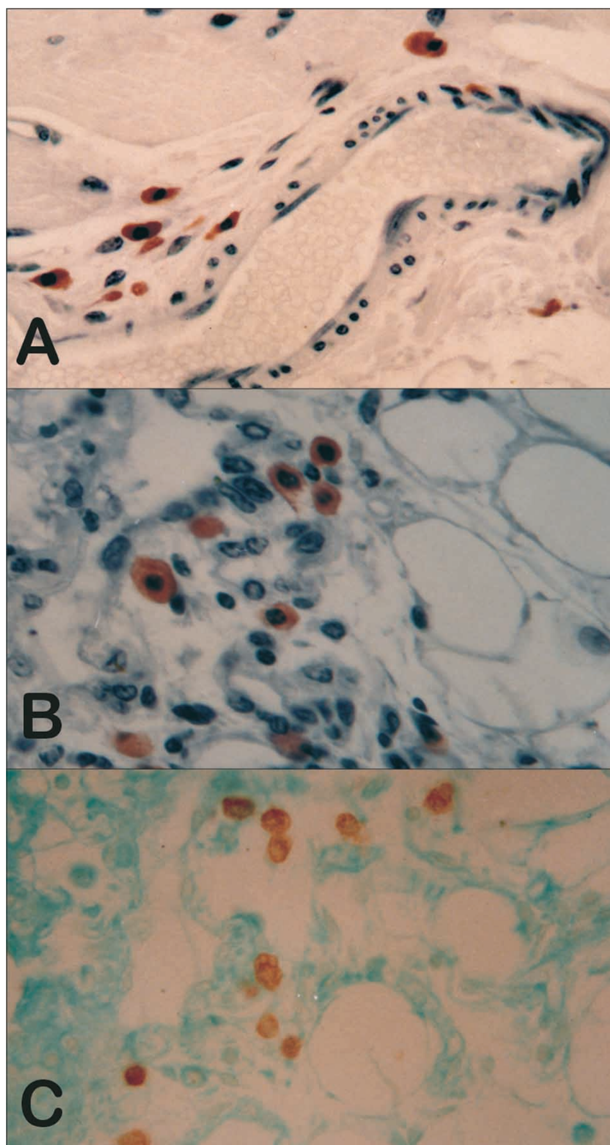

Fig. 1. A) Section of a hamster cheek pouch and B) lung: ferroin-hematoxylin staining. C) Section of lung counterstained with light green. Final magnification ×400.

blue O stained sections, and 12.94 ± 1.4 for alcian blue-safranin O stained sections. Differences between groups were not statistically significant (ANOVA; $p > 0.05$).

Discussion

Phenanthrolines constitute a group of heterocyclics that are employed as metal reagents in analytical chemistry (Lillie 1977). Ferroin (Fig. 3) is the colored co-ordination complex (metallochrome) of ferrous iron with *o*-phenanthroline. The complex contains iron (II) tightly bound by a polar covalent bond to the nitrogen atoms of *o*-phenanthroline. In this way, and similar to the process involved in the formation of iron hematoxylin, alcian blue 8G, iron-eriochrome cyanine R or gallocyanine-chrome alum, a colored cationic complex is formed whose point charge favors its binding to the polyanions in the tissue (Prentø 2001, Kiernan 2001, Horobin 2002).

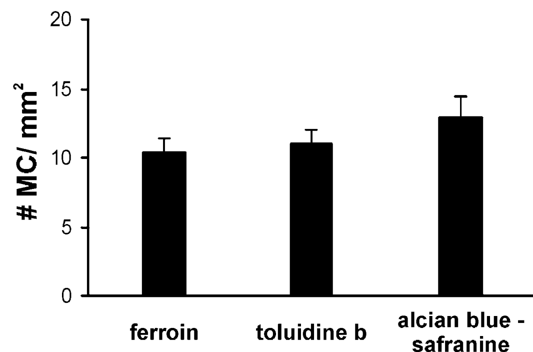

Fig. 2. Mast cell counts employing each of the three staining procedures. The data are expressed as mean ± SEM (ANOVA; $p > 0.05$).

Hukill and Putt (1962) employed a diphenylated derivative of *o*-phenanthroline (*batho*-phenanthroline) for detecting tissue iron. Ferroin has been used in several spectrophotometric studies and as a chelating agent of bivalent metals in different biological and chemical assays (Vydra and Kopanica 1963, Schmidt et al. 1981, Allen et al. 1983, Issopoulos 1992, Dunn et al. 1993, Xu et al. 1996, Boumans et al. 1997, Luque-Pérez et al. 2000). The use of ferroin for histochemical studies, however, has not been reported previously.

The granules of mast cells contain different chemical mediators. Heparin, one of the principal mediators, is a heteropolysaccharide (glycosaminoglycan) with a long chain of repetitive disaccharide units that contain at least one or two negative charges afforded by sulfate and/or carboxyl groups. These negatively charged heteroglycans

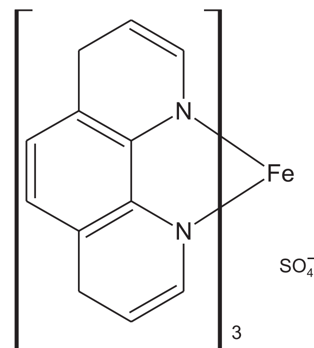

Fig. 3. Ferroin structure (no C.I. number).

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can be stained by planar, low molecular weight, monovalent basic dyes such as toluidine blue O (CI 52040) and pyronin Y (CI 45005), by polyvalent dyes such as alcian blue (CI 74240), or by large cationic staining species including colloidal iron, cationized ferritin, and high iron diamine (Horobin 2002).

The results reported here show that ferroin is a colored cationic complex with highly specific staining affinity for mast cell granules. These findings indicate that staining results from covalent bonding between carboxyl and/or sulfate groups of heparin and the ferroin by electrostatic bonding (Horobin 2002). Thus, the negative charge density of those groups in the heteroglycans is very large and confers on these molecules a great capacity to bind water and counterions (Prentø 2001) such as those of ferroin.

The use of prolonged staining times for ferroin resulted in background staining. Staining times exceeding 30 min resulted in faint staining of cartilage, hair, and keratinized epithelia. Staining became stronger as staining time increased. In this regard, our results agree with those of Horobin (2002), who stressed that long staining times lead to labeling of additional polyanions such as DNA. Despite the fact that ferroin can stain heteroglycans in general, the selectivity of mast cells staining is influenced by the rate of stain entry given that the differential result is time-dependent (Prentø 2001, Horobin 2002). Thus, the ferroin technique stains mast cells selectively by intentionally controlling stain diffusion. Shorter than optimal staining times result in faint staining of mast cells.

Miyata and Takaya (1980) reported that samples fixed with agents that contain aldehydes do not alter the staining properties of mast cells granules. Despite the fact that we have not performed a study comparing the influence of different fixatives on mast cell staining, we observed that fixation with formaldehyde alone or formaldehyde plus cetylpiridinium (0.5%) did not alter the staining properties of the heteroglycans exposed to ferroin.

The method described here is simple and can be applied to archival material. Admittedly, the ferroin technique fails to distinguish the different functional populations of mast cells, whereas the alcian blue-safranin O method does allow these distinctions. The advantage over other staining techniques, however, lies in the possibility of staining nuclei with hematoxylin or of choosing a counterstain to enhance cell-tissue contrast thus improving localization of mast cells within tissues. Provided contrast is adequate, the method is particularly useful for quantitative evaluation of mast cells by image analysis, thus allowing the assessment of a significant number of samples in a short time.

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