Human intestinal mucosal mast cells: evaluation of fixation and staining techniques

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SUMMARY The staining properties of tissue mast cells are influenced by the method of fixation. Differences in fixation and staining techniques may explain the contradictory results in the published reports on the number of human mucosal mast cells (MMC) in the gastrointestinal mucosa in health and disease.

We have examined the influence of fixatives on the staining properties of human MMC in operative biopsy specimens of human jejunum. Specimens were divided into pieces, each of which was fixed in one of the following fixatives: Carnoy's, basic lead acetate (BLA), Baker's, Bouin's, isotonic formol-acetic-acid (IFAA), 10% neutral buffered formalin, formol sublimate, and formol saline. Thereafter, tissues were paraffin-embedded and 5 µm sections were cut and stained with either astra-blue/safranin pH 0.3, or toluidine blue pH 0.5. Counts of the number of MMC/mm² were obtained for each fixation method. The results show a critical influence of the fixative on the number of mast cells identified after staining. For example with astra-blue/safranin the mean MMC/mm² count was 40 in formol-saline-fixed specimens, and 268 in Carnoy's-fixed specimens. In biopsies fixed with formalin-based fixatives, mast cells were more readily stained with toluidine blue. It is recommended that Carnoy's or BLA be used as the fixative for any light microscopic study of human MMC.

The mucosa of the gastrointestinal tract contains many lymphoid cells. These include lymphocytes, plasma cells, macrophages, mast cells and eosinophils. Mucosal mast cells (MMC) and IgE-mediated immune reactions, are of interest not only in view of their established role in parasite immunity,¹ ² but also because reaginic hypersensitivity, or other immunological reactions involving mast cells, may be implicated in a range of intestinal hypersensitivity diseases. For clinical diagnosis, and in research studies, various methods are used to stain mast cells in human mucosal biopsies, and such biopsies have generally been fixed in a formalin-based fixative before processing. Yet it is known from studies in animals that staining patterns of MMC are critically dependent upon the fixation technique. In 1966 Enerbäck³ showed that the rat small intestine, when fixed in 4% formaldehyde, appeared to be completely devoid of MMC whereas many such cells could be detected with other fixation methods. We are not aware of a comparable study of the optimal fixation and staining methods for MMC in man. We have therefore carried out an experiment, using operative intestinal biopsies and a range of commonly used fixatives, to establish whether the method of fixation influences the number of MMC detected in human intestinal mucosa.

Material and methods

Operative biopsies of normal jejunum were obtained from 13 patients who were undergoing abdominal surgery. In all cases the intestine was incised for resection or for creation of an anastomosis. Diagnoses were pyloric stenosis 4; adenocarcinoma of stomach or colon 3; duodenal ulcer 3; gastric ulcer 1; adhesions 1; unexplained abdominal pain 1. Each specimen was rinsed in cold saline and divided into eight pieces which were placed in fixative within 10 min. The period of fixation varied from one to seven days (except for Carnoy's) as indicated below. After fixation the tissues were rinsed in cold saline and divided into sections, 5 µm thick, were cut and stained with astra-blue/safranin and with toluidine blue.

Fixatives³ ⁴

1 Carnoy's fixative (60 ml absolute ethanol, 30 ml chloroform, 10 ml glacial acetic acid). Mini-
mum fixation time 2 h.

2 BLA (Mota's basic lead acetate acetic-acid ethanol, 1 g basic lead acetate, 50 ml ethanol, 50 ml distilled water, 0.5 ml glacial acetic acid). Minimum fixation time 24 h.

3 IFAA (isotonic formol-acetic-acid, 1-5% formalin (commercially available solution of 40 g formaldehyde in 100 ml H2O, 0.5% glacial acetic acid). Minimum fixation time 2 days.

4 Baker's fixative (formalin calcium chloride, 10% formalin, 2% calcium chloride in distilled water). Minimum fixation time 24 h.

5 Bouin's fixative (25 ml formalin, 75 ml saturated picric acid solution, 5 ml glacial acetic acid). Minimum fixation time 24 h.

6 10% buffered formalin pH 7-0 (10 ml formalin, 90 ml tap water, 0.4 g NaH2PO4. 2H2O and 0.65 g Na2HPO4 added to give pH 7-0). Minimum fixation time 7 days.

7 Formol sublimate (10 ml formalin, 90 ml saturated aqueous mercuric chloride solution). Minimum fixation time 24 h.

8 Formol saline (10 ml formalin, 90 ml 0.85% sodium chloride). Minimum fixation time 7 days.

STAINS

Astra-blue/safranin pH 0-35
Astra-blue powder (1-0 g) (Gurr Chemical Ltd, Code Number 2870) was dissolved in 100 ml 0-7 N HCl. Concentrated HCl was added to lower the pH to 0-3 which gave a final concentration of about 0-8 N.

Sections were stained in 1% astra-blue solution for 30 min, and washed thereafter for 5-10 min in 0-7 N HCl. Counterstaining was achieved by dipping the sections into 0.5% safranin 0 (Gurr Chemical Ltd, Code Number 27300) in 0-125 N HCl for 30 s or less. Finally the sections were taken through graded alcohols to xylene and mounted in coverbond TM (Harleco-Herstal, Liège, Belgium, Code Number 7886).

Toluidine blue pH 0-54
Toluidine blue powder (0-5 g) (Gurr Chemical, Code Number 29880) was dissolved in 100 ml 0-5 N HCl (pH 0-5). The sections were taken to water as above and were stained with this solution for 30 min. They were rinsed in water for 5-10 min, differentiated in 95% alcohol, and were cleared and mounted as above.

METHOD FOR MAST CELL COUNTS
Mast cells were counted in well orientated sections cut perpendicular to the mucosa and in which the muscularis mucosae was intact. Counts were performed on coded slides on a Leitz Dialux 20 EB microscope (eyepiece × 10, objective × 40). A 10 mm² eyepiece graticule, calibrated against a calibration slide was used. The edge of the graticule was orientated along the muscularis mucosae, at the base of the crypts. The area covered by the square of the graticule comprised 80-100% of the total depth of the mucosa (from the bottom of the crypt to the tip of the villus) (Fig. 1). On each slide, eight to ten fields (290 μm × 290 μm; area 0-084 mm²) were counted and the MMC count per specimen was expressed as MMC/mm². No attempt was made to correct for the area covered by epithelium.

STATISTICAL ANALYSIS
Results are expressed as mean (±) and standard deviation (SD) or standard error of the mean (SE) and were compared by Student's t test. The reproducibility of duplicate counts on the same slide was assessed by linear regression analysis.

Results
Of the specimens obtained from the patients, 208 pieces of tissue were available for study, and the orientation of 201 samples was such that enumeration of MMC could be carried out. Mast cells were seen both in the mucosa and submucosa. Those in the submucosa were typical of other connective tissue mast cells and contained many intensely stained granules which frequently obscured the nucleus. The MMC in the mucosa were smaller and contained fewer granules. This difference between mucosal and submucosal mast cells has also been reported in the rat. Granulated lymphocytes of a type commonly found in rodents were detected with the oil immersion objective (× 100). They were located intraepithelially in small numbers in many of the
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specimens examined and were unlikely to be confused with the rarer intraepithelial mast cells in the epithelium. Granulated intraepithelial lymphocytes were medium sized, having light, rather featureless cytoplasm which contained smaller and fewer granules than MMC; these were located peripherally along the cell membrane and did not obscure the nucleus. The staining properties of lymphocyte and MMC granules were, however, similar.

There was considerable variation in the background staining of tissues, depending on the type of fixative used (illustrated in Fig. 2) but this was not the reason for the marked differences in MMC counts described below. MMC stained with astrablue/safranin had blue granules against a pale red background. When stained with toluidine blue, the granules were deeply stained with a violet metachromasia against a pale blue background. The background staining in formalin-fixed biopsies was more intense than in tissues fixed in Carnoy's or BLA fixative. In Carnoy's and BLA-fixed tissues, the mast cells were extremely well preserved and could be detected at all levels of the lamina propria, including the villus core (Fig. 2) and, rarely, the crypt epithelium. In tissues fixed in formalin, MMC were predominantly located in the basal mucosa and were poorly preserved, as was evident from the swollen appearance of the cells and from the virtual absence of mast cells in the villus core.

For photographic documentation of MMC, preparations stained with toluidine blue were found to give better black and white contrast than did those stained with astra-blue/safranin.

**Evaluation of counting technique**

All MMC counts were made on coded slides with one edge of the graticule orientated along, and covering, a 290 μm length of muscularis mucosae. Formalin-based fixatives produced considerable shrinkage of the biopsies and thus the graticule depth consistently included 100% of mucosal depth in these preparations. In contrast, between 80 and 100% of the depth of the mucosa was covered by the graticule in tissues preserved in Carnoy's or in BLA (see Fig. 1). A preliminary evaluation of this differential shrinkage effect as a source of error in MMC counts revealed that most MMC were confined to the lower one third of the mucosa. With non-formalin-based fixatives, less than 2% of MMC in an area of mucosa of 0.084 mm² would be missed by applying the method described above. Thus this technique is basically a tissue unit counting method as used with minor variations by several investigators.8–12

All counts were made by one investigator (SS).

**Fig. 2. Illustration of the critical effects of fixative on the staining properties of human mucosal mast cells.** An operative biopsy of normal jejunum was fixed in Carnoy's (Figs. 2a, b), Bouin's (Figs. 2c, d), and 10% buffered formalin (Figs. 2e, f) tissues were paraffin-embedded and 5 μm sections were cut and stained with toluidine blue.

**Fig. 2a.** Many mast cells are clearly seen as dark cells against a pale background stain. MMC count: 190/mm²; Carnoy's, toluidine blue × 190.

Duplicate counts in 12 specimens showed a correlation coefficient of r = 0.917 (p < 0.001) by linear regression analysis, confirming the internal consistency and reproducibility of the technique.

**Mast cell counts**

The number of mast cells detected in tissue sections was found to depend critically on the type of
Fig. 2b Higher magnifications (× 600) demonstrate the typical granular appearance of mast cells and its good preservation (arrow).

Fig. 2c Some mast cells can be seen in the villus core, and there are well stained mast cells in the submucosa. MMC count: 100/mm²; Bouin’s, toluidine blue × 190.

Fig. 2d At higher magnification (× 375) the granular appearance of MMC and of some intraepithelial cells is noticeable (arrow).

fixative employed. In order to apply statistical analysis to this subjective observation, the counts for each fixative/stain combination made on the 13 biopsies of normal jejunum have been combined, and mean, SD (not shown) and SE calculated. (Thus each group consisted of 11-13 biopsies for each of the 26 fixative/stain combinations.) The results are shown in Fig. 3.

Substantially more MMC were detected in tissues which had been fixed with Carnoy’s or with BLA, than in the tissues fixed in formalin-based fixatives. For example, after staining with astra-blue, the mean value for the 13 specimens was 268 MMC/mm² in Carnoy’s fixed tissue, and only 47 MMC/mm² in biopsies fixed in 10% buffered formalin (p < 0.001). That the pattern was consistent within each biopsy is illustrated in Fig. 4. There were 11 biopsies in which counts for Carnoy’s, Bouin’s, and buffered formalin-fixed preparations were available, and in every case fewer MMC were detected when Bouin’s was compared with Carnoy’s, and when buffered formalin was compared with Bouin’s.

Statistical analysis of the various results is summarised in the legend to Fig. 3. When compared to the MMC/mm² in Carnoy’s fixed specimens, significantly (p < 0.001) fewer mast cells were
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Fig. 2e Only occasional mast cells are found in villus core and around the crypt (arrow), although submucosal mast cells (arrow) are well stained. MMC count: 68/mm²; 10% buffered formalin, toluidine blue × 190.

Fig. 2f At higher magnification few poorly preserved MMC (arrow) are demonstrated, whereas the submucosal mast cells are well preserved and stained × 375.

present in samples fixed in any of the formalin-based fixatives. Mast cell counts were comparable whether sections were stained with astra-blue or toluidine blue, except where the tissues were fixed in formalin-based fixatives where counts tended to be lower with astra-blue staining, and this difference was significant (p < 0.025) in the 10% buffered formalin-fixed tissues.

Discussion

This study demonstrates unequivocally that the preservation of MMC in human intestinal mucosa is a function of the type of fixative employed. Thus, significantly more MMC were detected after fixation in Carnoy's or BLA than in fixatives containing 10% formalin. These observations may explain the widely discordant reports of intestinal MMC counts in human disease (Table).13–18 Most published studies have been performed in formalin-fixed preparations, and this study has demonstrated that fivefold higher mast cell counts can be obtained, in normal tissues, if Carnoy's is used as the fixative.

Although the composition of human MMC granules has not been examined in detail they are likely to have many features in common with the granules of rat mucosal mast cells. The latter contain monoamines,1,3 basic protein,1 a protease,19 and acid mucopolysaccharides (glycosylaminoglycans, GAG).3 In the rat, MMC GAG are less strongly sulphated than connective tissue mast cell GAG.1,3,20

The staining methods used to demonstrate mast cells by light microscopy depend on the affinity of cationic dye for the mast cell granule GAG. Optimal fixation will result in precipitation of GAGs leaving polyanionic sites available for binding of dye. Failure to demonstrate mast cells in fixed tissues may be due either to dissolution of nonprecipitated GAG,3 or to blocking of polyanions by cationic
proteins. Experimental data favour the latter hypothesis since rat mucosal mast cells can be demonstrated in formalin-fixed intestine by the critical electrolyte concentration technique. Carnoy's and lead-based fixatives penetrate tissues rapidly, and precipitate both GAG and proteins; also the acidity of Carnoy's may facilitate ionic linkages between GAG and basic cationic dyes.

In virtually all of the specimens examined, as is illustrated in Fig. 2, submucosal mast cells were found to be much less sensitive to the effects of fixation than were MMC. Such differences between mucosal and submucosal mast cells have long been recognised, one of the first reported observations being by Maximow in 1906 (for review). However, it is not known if human MMC, like those of the rat, differ from submucosal or connective tissue mast cells in their response to amine-releasing stimuli.

Metachromatic granules are present in the cytoplasm of many of the small intestinal intraepithelial lymphocytes of a variety of species. Some authors indeed use the term "granular lymphocyte" to describe the intraepithelial lymphocyte, and it has been suggested that these cells are the precursors of mast cells. An important finding in our study has been the observation that a small proportion of human mucosal intraepithelial lymphocytes do contain metachromatic granules as has been frequently found in rodents. Judging from their staining properties, the granules of intraepithelial lymphocytes are likely to contain GAG but, when compared with MMC, lymphocytes contain smaller and fewer granules which do not obscure the nucleus. Whether these distinctive populations of granulated lymphocytes in human gut represent, as is postulated for rodents, a bone marrow-derived T cell-independent or a T cell-dependent subpopulation remains to be established.

Research on MMC in patients will probably be pursued in conditions such as inflammatory bowel disease, allergic intestinal disease and infectious diseases. For such work the pathologist is likely to include counts of eosinophils and of IgE-containing

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**Fig. 3** Influence of eight fixatives and two stains on the MMC count in operative biopsies of normal human jejunum. With astra-blue/safranin stain, mean MMC count was highest for Carnoy's-fixed specimens. In tissues fixed with IFAA, Baker's, Bouin's and the three 10% formalin based fixatives, MMC counts were significantly lower (p < 0.001) than in Carnoy's-fixed tissues. With toluidine blue stain, mean MMC count was again highest in Carnoy's fixed specimens. In tissues fixed with Baker's, Bouin's, and the three 10% formalin-based fixatives, MMC counts were significantly lower (p < 0.001) than in Carnoy's-fixed tissues. With this stain the difference between IFAA-fixed specimens and Carnoy's was significant at p < 0.05. With astra-blue and toluidine blue stains, MMC counts in BLA-fixed preparations were lower than in Carnoy's-fixed preparations, but the differences were not significant.

Comparison between the two stains showed that values for MMC counts were higher in toluidine blue stained sections, in those specimens which had been fixed in the formalin-based fixatives. This reached significance only for 10% buffered formalin (p < 0.025).

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**Fig. 4** MMC counts in 11 operative biopsies of normal human jejunum, fixed in Carnoy's, Bouin's, and buffered formalin and stained with toluidine blue. All specimens showed the same pattern, with MMC counts being consistently lower in Bouin's and buffered formalin-fixed preparations.
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**Published results of mucosal mast cell counts in human jejunum**

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*CMPI = cows' milk protein intolerance.

plasma cells—for example, by an immunoperoxidase technique, in the protocol. Carnoy's fixed specimens are unsuitable for the staining of eosinophils by carbolchromotrop 2R, and immunoperoxidase staining techniques are in general optimal in formol saline or formol sublimate-fixed materials. Thus, if light microscopic evaluation is to be used, there is at the moment no alternative but to divide a biopsy specimen for separate processing for mast cells and for IgE/eosinophil counts.

We recommend that the standard method for processing of human mucosal biopsies to evaluate intestinal MMC should be the use of Carnoy's or BLA as fixative, and astra-blue/safranin or toluidine blue as stain.

Not all commercially available batches of astra-blue dye are suitable for staining of mucosal mast cells. We have obtained excellent results with Gurr's astra-blue (which is unfortunately no longer available). Similar good results have been obtained with astra-blue from E Merck, Darmstadt, FR Germany.

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