Rhinology, 27, 81-89, 1989

Mast cell distribution and morphology in human nasal turbinates following decalcification

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SUMMARY

Whole inferior nasal turbinates were used to evaluate six different fixatives followed by decalcification. H and E staining was used to assess general fixation and toluidine blue and thionin to stain mast cells metachromatically. We show that neutral buffered formalin or paraformaldehyde give the best overall fixation. Very long times (six days) are needed in TB to show maximum mast cell numbers; these numbers are never as high, nor the cells as densely stained as those in undecalcified controls. The difference in number between the two supports the hypothesis that there is more than one subset of mast cells in nasal mucosa. The more superficial mast cells have a smaller size and may be dendritic, with relatively few granules which stain faintly. Those in deeper situations are often larger, ovoid, very granular and intensely stained.

INTRODUCTION

Nasal mucosa provides one of the most readily accessible sources of human mast cells. Despite the great importance of mast cells in mechanisms of allergy, most studies have been on either scrapings, small punch biopsies or secretions (Okuda et al., 1985; Otsuka et al., 1985). Studies have been done on human mast cells in other tissues, e.g. gastro-intestinal tract (Strobel et al., 1981), bronchi (Ts'ao et al., 1977), bronchiole (Trotter and Orr, 1973) and skin (Trotter and Orr, 1974). These all show the differences between human and other species and emphasise the need for more work to be done on human tissue.

A review of the literature on mast cell fixation in the last twenty years shows that a wide variety of fixatives have been used, with very variable results. Because we could readily obtain whole inferior turbinates directly following surgical removal, it was decided to survey a number of commonly used fixatives to assess the optimal technique. Even careful dissection of the soft tissue from the underlying bone tends to destroy the deepest layers so it was decided to fix slices of mucosa still attached to the bone. A small amount of mucosa was dissected off the

bone and processed as an undecalcified control, for comparison with previous reports of mast cells in small samples of superficial nasal mucosa. Our aim was to obtain optimal fixation of the tissue as a whole while demonstrating maximal numbers of mast cells and retain the ability to identify other cell types.

MATERIALS AND METHODS

Eight patients with chronic rhinitis, three males and five females, ranging in age from 16 to 53 years (mean 33.9 years) were studied. Two were atopic (one or more positive reactions to standard skin prick tests), and six were non-atopic. The nose was packed with ribbon gauze soaked in 2 ml of 10% cocaine for 10 minutes pre-operatively. Immediately following removal, the turbinates were swabbed free of blood and mucus and cut into transverse slices of 2–3 mm. Within five minutes of removal they were put into one of the following fixatives (Hopwood, 1982, unless otherwise stated):

1. Neutral buffered formalin (NBF): 24 hours: room temperature.
2. Paraformaldehyde (PFA): 6 hours: 4° C; (Huntley et al., 1985).
3. Mota's basic lead sub-acetate (MBLSA): 24 hours: room temperature (Strobel et al., 1981).
4. Bouin's solution (BS): 6 and 24 hours: room temperature.
5. Carnoy's solution (CS): 1 and 24 hours: room temperature.
6. Baker's formal calcium (BFC): 6 and 24 hours: room temperature.

After fixation, tissue was decalcified by one of the following methods:
A. Formic acid 50%: sodium citrate 20% mixed in equal volumes (J. Cable, personal communication). Decalcified for 7–10 days, changing solution every two days. Used to follow fixatives 1–4 and 6.
B. Jenkins' solution: following fixation in 5 (Culling, 1957) for the same times as (A).
Following decalcification, all tissue was dehydrated through graded ethanols for three hours each and cleared overnight in Inhibisol® prior to embedding in Paraplast® at 60° C for 3 x 1 hour and then transferred to fresh Paraplast® and cooled. Serial sections were cut at 6μm and mounted on glass slides. They were dewaxed using xylene and rehydrated through ethanols to tap water. Following staining, sections were dehydrated again, cleared in xylene and mounted in Depex Mounting Medium.

Staining methods

1. Haematoxylin and eosin (H and E):
Sections were stained in Gill's haematoxylin (Gill et al., 1974) for 10 minutes, differentiated in 1% HCl in 70% ethanol for 15 seconds and in tap water for 5 minutes, followed by counterstaining in 1% eosin for 5 minutes (Stevens, 1982).

2. Toluidine blue (TB); pH 0.5 (Huntley et al., 1985):
Sections were stained for 15 minutes, 3 hours, overnight (17 hours) and 6 days.

3. Thionin (Bancroft and Stevens, 1982):
An increased staining time of 50 minutes was used, followed by brief differentiation (about 5 seconds) in 0.2% acetic acid.

Cell counts

Mast cell counts were done using a rectangular photographic eyepiece, calibrated with a calibration slide, on a Leitz Laborlux K microscope at x 400. For the superficial counts, the short edge of the eyepiece was orientated along the basement membrane and fields containing predominantly connective tissue (i.e. no glands or vessels) were selected in an attempt to standardise between specimens. Care was taken not to overlap any two fields. Deep counts were made by similar means with the graticule moved over deep tissue parallel to the superficial tissue, again ensuring that no overlapping took place. For each superficial and deep count 15 fields were counted. When serial sections from the same block were counted, fields that were as comparable as possible were counted.

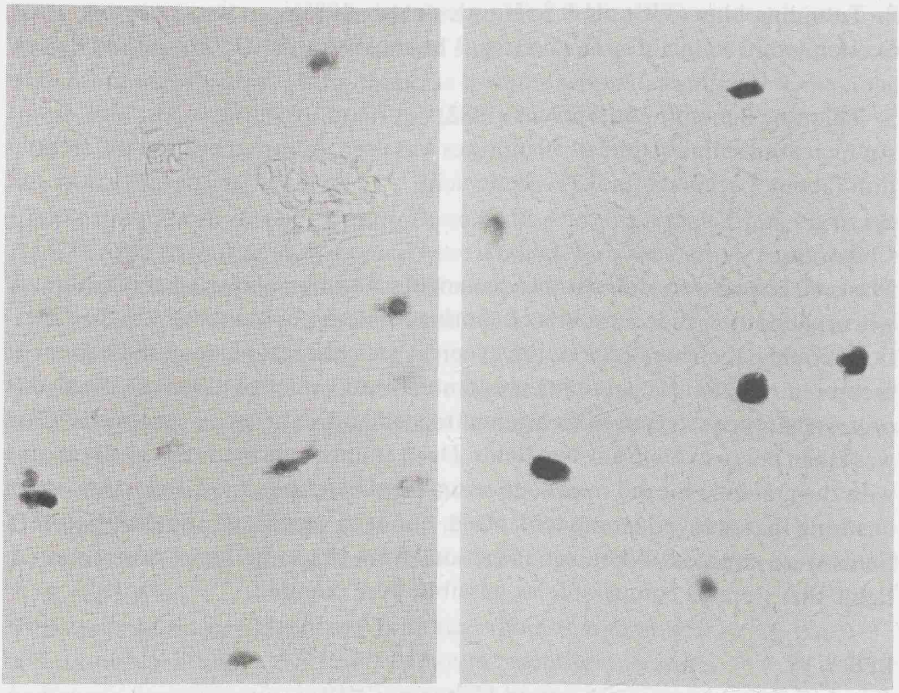
RESULTS

Decalcified tissue

1. Haematoxylin and eosin:
Sections stained with H and E were used to evaluate the general tissue morphology. Those fixed in NBF or PFA showed the best overall preservation. Different cell types were readily identifiable and there was little evidence of distortion or artefacts. Examination of BS and BFC fixed tissue showed that the 24 hour time resulted in better preservation than the six hour time, and fixation was good. Tissue fixed in MBLSA and CS for one hour was poorly preserved, with large areas that stained unsatisfactorily, especially in the deeper tissue. CS for 24 hours showed improved fixation over one hour, but identification of some cell types was difficult.

2. Toluidine blue:

A. *Distribution of mast cells:* Mast cells were seen as granulated cells of variable size and shape (Figure 1). In the two atopics it was noticeable that within the epithelia there were some mast cells, whereas they were not seen in non-atopics. Most mast cells were seen in the lamina propria, just deep to the basement membrane and between or deep to the areas of glandular tissue and vessels. These cells tended to be small in size with several dendritic processes and a moderate number of granules, with the nucleus usually visible. More deeply located mast cells tended to be larger in size and rounder or ovoid in shape, with many



a b

Figure 1. Typical mast cells in (a) superficial nasal mucosa and (b) deeper tissue. Fixed in NBF and stained in toluidine blue for six days; x480.

granules that often obscured the nucleus. Not all mast cells conformed to this distribution; sometimes large, very granular cells were seen superficially with some dendritic cells in the deeper tissue.

B. *Numbers of mast cells:* The numbers of mast cells in the different samples were counted after different times in stain. Numbers from one individual, which are typical, are shown in Table 1. In all samples the staining intensity of the mast cells increased with time. After counting cells in superficial and deeper areas, it was noted that the increase was in the superficial tissue. Only in CS did the mast cell numbers stay almost constant.

C. *Stain quality:* As well as counting cells, we assessed the quality of the staining. The mast cells exhibited violet metachromasia of the granules and small numbers were easily identifiable against a pale blue background after 15 minutes' staining following all methods of fixation. Although numbers increased, the background stain decreased very markedly with time, making it impossible to identify any tissue component other than mast cells, thus making assessment of their distribution almost impossible. We found that keeping rinsing and dehy-

Table 1. Superficial mast cell numbers in nasal mucosa from one individual after different fixation and times in TB.

time	fixative					
	NBF	PFA	MBLSA	BS	CS	BFC
15 minutes	30	29	1	39	53	19
3 hours	37	38	11	49	65	40
17 hours	44	56	16	59	59	43
6 days	70	62	31	67	62	65

dration times to a minimum retained at least a little of the background. CS fixed tissue never had any background stain. Mast cells in MBLSA and CS fixed tissue always tended to be pale, with the cells having a dendritic shape and few granules; very few large oval cells were seen in these.

3. Thionin:

Thionin staining proved to be very unreliable. The spectrum of mast cell staining ranged from red-purple metachromasia in NBF and PFA fixed tissue, to a dark blue colour in CS tissue and a much more intense dark blue in BS and BFC fixed tissue. MBLSA tissue had no demonstrable mast cells with this method. Thus metachromasia could be demonstrated consistently with only NBF or PFA. The cells also appeared rounded rather than dendritic and the deeper ones stained much better than the more superficial ones.

Undecalcified tissue

Toluidine blue staining was done on PFA fixed tissue which had been dissected off the underlying bone and not decalcified. Here, mast cells stained much more intensely than in the decalcified tissue from the same individual. Cell numbers remained relatively constant no matter how long the tissue was stained (Figure 2), with larger numbers of mast cells than in comparable decalcified tissue.

DISCUSSION

This is the first systematic survey of nasal mast cells in whole turbinate specimens and the first report of the effects of decalcification on mast cell counts. In this study we have evaluated which fixative gives:

1. the best overall preservation of the tissue as shown by H and E stain, and
2. maximum numbers of mast cells when followed by decalcification and TB staining.

Tissue preservation

The H and E results show good preservation in NBF and PFA, and also in BS and BFC at the longer fixation time. Both MBLSA and CS are poor. All the fixatives

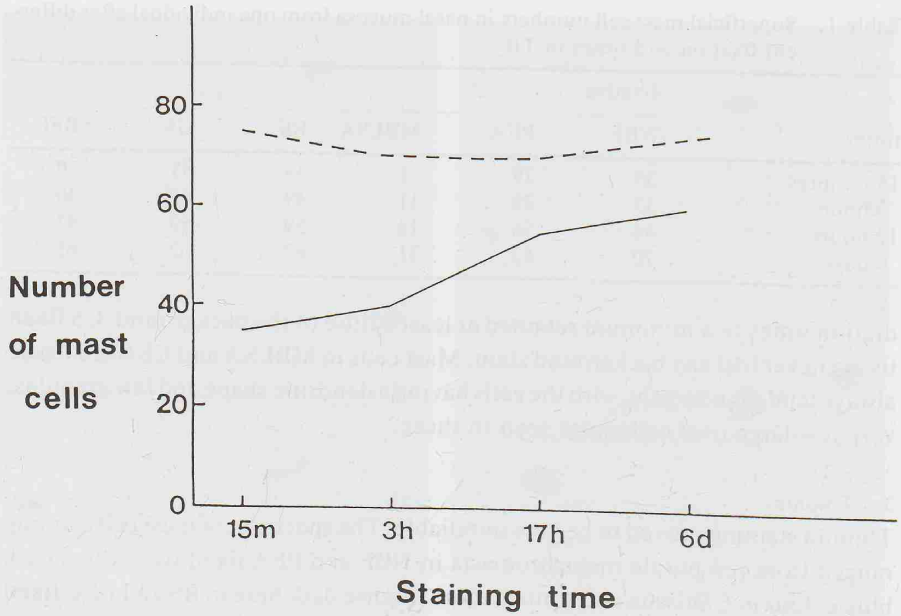


Figure 2. Effect of decalcification on mast cell counts at various staining times; PFA fixed _____ decalcified tissue;
_ _ _ _ non-decalcified.

that produce good preservation are formalin based and bring about fixation by cross-linking of protein end groups. BS also contains picric acid and this fixes by precipatating protein and also bonds with surface groups of some proteins (Pearse, 1980). The MBLSA contains lead salts, which Curran (1964) states cause precipitation of acid mucopolysaccharides (now called glycosaminoglycans or GAGs) and proteins. Szirmai (1963) related the fixative effect of CS to its acidity which facilitates ionic linkages between GAGs and proteins.

The tissues in this study were also subjected to decalcification in mild acid solutions over several days. The fixatives containing formalin show optimal preservation after the decalcification which may be due to the cross-linking of proteins protecting against the effect of the acid. This is despite the fact that the formalin may have been partially washed out following only 24 hours' prior fixation.

CS has become a standard for the fixation of mast cells and has been used in many studies, amongst them those of Tas and Berndsen (1977), Miyata and Takaya (1980), and Strobel et al. (1981). Following decalcification, it still gave high counts but is unsatisfactory because of poor background staining and weak mast cell staining. Because of this, and the problem of cell identification following H and E staining, we would not recommend CS as the fixative of choice.

Mast cell counts

In 1966, Enerback showed that the most critical step in mast cell demonstration is the fixation. He noted the failure of TB staining in rat intestinal mast cells following formalin based fixation and concluded that this was because the fixative had extracted some of the granule contents. In 1983, however, Wingren and Enerback showed these cells could be demonstrated providing they were stained for long enough, i.e. three days, and that trypsinisation reduced the time required. Because mast cells in tongue and skin stained after only a short time, it was suggested that the intestinal cells had a protein "shell" around the granule content which, when cross-linked by the formalin, would prevent dye penetration.

All mast cell granules consist of a GAG component ionically bonded to proteins (Lagunoff, 1966). GAG and proteins, such as histamine and serine esterase, can be extracted from rat peritoneal mast cells (Lagunoff and Pritzl, 1976). The GAG may be heparin or a less sulphated compound such as heparan sulphate (Tas and Berndsen, 1977), which are anionic. The protein component has cationic groups such as primary amino groups of lysine and guanidium groups of arginine (Wingren and Enerback, 1983).

When mast cells are fixed in an aldehyde, it combines with the protein. Formalin contains a number of impurities which induce cross-linkage of the proteins (Pearse, 1980), thus making a "shell" around the granule and rendering the GAGs relatively inaccessible to dye molecules. Paraformaldehyde, which is purer, forms fewer cross-links and so forms a less complete "shell".

The majority of our samples were decalcified by acid which could act on the tissue to extract some of the GAGs and protein. This would explain the results obtained from our formalin fixed samples stained in TB, i.e. all show increased mast cell numbers with time and relatively pale staining of the cells. Although the formalin induces the "shell" around the granules, the acid extracts some of that protein along with some GAGs. The dye penetrates the granule with relative ease, but there is less GAG available to stain, so the stain is less intense.

The study of Otsuka et al. (1985) yielded much higher numbers of mast cells in nasal mucosa fixed in MBLSA than in formalin. Their tissue samples were smaller than those used in this study, they were fixed for a shorter, but variable, time and were not decalcified. The embedding in plastic followed by a different stain formula to ours means the techniques are not absolutely comparable. Nonetheless, we would not disagree with the conclusion that there is more than one subpopulation of mast cells in the nose. One is the more deeply located, large, heavily granulated type which fixes satisfactorily in formalin and stains in a short time. The other is the formalin resistant type which is slow to stain and is more superficially located and dendritic in shape. The heterogeneity of human mast cells was noted in ultrastructure studies by Trotter and Orr in respiratory tract mucosa (1973) and in skin (1974). Lee et al. (1985) highlight this heterogeneity in

their review; even within nasal mucosa there may be several subsets of mast cells. McKenna (1974), in his description of "nasal mastocytosis", denied the need to count mast cells which he thought to be uniformly distributed throughout the mucosa. Enerback et al. (1986) also state that mast cell distribution is even throughout the mucosal connective tissue stroma in small biopsy specimens taken before the pollen season. We find the majority of the cells located superficially and that their distribution is far from uniform. This finding suggests that the results of mast cell studies in small samples of nasal tissue must be interpreted with some caution. In the two atopic patients in our study, we observed mast cells in the epithelium which is similar to the report of Enerback et al. (1986). The control tissue which is PFA fixed and not decalcified has larger numbers of mast cells at all times than the decalcified sample. Figure 2 shows numbers from one typical individual. This suggests that the decalcification is removing GAGs from a small proportion of superficial cells, which again raises the possibility of more than one subpopulation of mast cells.

We conclude that NBF and PFA gave best overall tissue and mast cell preservation but that to demonstrate mast cells after decalcification very long staining times must be used. Thionin staining is not reliable and cannot be recommended under these conditions. This study has revealed interesting differences between mast cell groups in different areas of nasal mucosa but unless decalcification is considered essential to study the most deeply located mast cells, better staining and higher counts can be achieved using tissue that has not been decalcified.

ACKNOWLEDGEMENTS

This study was funded by a grant received by Dr. C.M. Trotter from the Sir Stanley and Lady Davidson Medical Research Fund, which is gratefully acknowledged. Skilful photographic assistance was given by Mr. Jack Cable.

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