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A comparison of methods for nasal mast cell demonstration

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SUMMARY

Nasal turbinates were studied from 14 rhinitis patients following surgical turbinectomy, and from five subjects at autopsy. Mast cell counts on turbinectomy specimens were compared following staining with toluidine blue or Alcian blue and safranin after fixation in either paraformaldehyde or neutral buffered formalin. Mast cell numbers were significantly greater in the superficial submucosa than in the epithelium or deep submucosa in both the rhinitis group and the autopsy subjects. The combination of PFA fixation and ABS staining gave maximum mast cell counts, revealed two morphological mast cell sub-types and gave optimal demonstration of nasal tissue. Nasal mast cells are thus not uniformly distributed, appear heterogeneous under light microscopy, are present in large numbers even in the elderly, and are best demonstrated using PFA fixation and ABS staining.

INTRODUCTION

Our previous preliminary study of a wide variety of histological techniques for use in nasal turbinates following decalcification indicated that optimum fixation was obtained by either paraformaldehyde (PFA) for six hours or by neutral buffered formalin (NBF) for 24 hours (Trotter et al., 1989). Mast cell demonstration was superior in undecalcified specimens. Although some fixatives yield high numbers of mast cells, for example Carnoy's fixative, the overall standard of tissue preservation was unsatisfactory. Previous reports of nasal mast cell numbers may not, therefore, have used optimum methods of tissue preparation (Otsuka et al., 1985; Okuda et al., 1985; Gomez et al., 1987) and have usually relied on small turbinate biopsies to assess numbers (Okuda et al., 1985; Enerback et al., 1986; Gomez et al., 1987).

The aims of the present study were a) to compare mast cell demonstration following PFA or NBF fixation in undecalcified nasal turbinates, b) to compare

mast cell counts following both fixatives using toluidine blue (TB) staining or Alcian blue and safranin (ABS) staining and c) to assess mast cell distribution and morphology in whole undecalcified nasal turbinate specimens.

MATERIALS AND METHODS

Whole inferior nasal turbinates were surgically removed from 14 patients with chronic rhinitis (seven males, seven females, aged 13 to 61 years, mean 30.3 years). Three were atopic, i.e. had a positive reaction to one or more prick skin tests. All patients had symptoms of longstanding nasal obstruction. Nine also had intermittent watery rhinorrhoea. 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 either:

1. PFA: 6 hours: 4°C (Huntley et al., 1985) or
2. NBF: 24 hours: room temperature (Hopwood, 1982).

Turbinates were removed from five autopsy subjects, three males and two females to obtain normal tissue. These subjects were considered to be the best available source of intact human turbinates in non-surgical patients. The age of this group was of course considerably older (75 to 87 years, mean 79 years) than the surgical group. The autopsy tissue was taken within 24 hours of death and fixed in PFA, as above. NBF was not used for the autopsy specimens which were studied after we had ascertained in the rhinitis group that PFA was the optimum fixative.

After fixation, tissue was dehydrated through graded ethanols for three hours, cleared overnight in Inhibisol® prior to embedding in Paraplast® at 60°C for 3 × 1 hour, transferred to fresh Paraplast® and cooled. Serial 6 μm sections were cut, mounted on glass slides, dewaxed using xylene and rehydrated through ethanols to tap water. Following staining, the sections were dehydrated again, cleared in xylene and mounted in Depex Mounting Medium.

Staining methods

1. TB; 0.5% at pH 0.5 (Huntley et al., 1985). Sections were stained for 30 minutes.
2. ABS; Method B according to Mayrhofer (1980). An initial pilot study was done using critical electrolyte concentrations of 0.06M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.9M $MgCl_2$. We showed that the best mast cell demonstration in our tissue is obtained by working with 0.3M $MgCl_2$ critical electrolyte concentration.

Cell counts

Nasal turbinate mucosa comprises three structurally distinct laysers; epithelium, superficial submucosa and deep submucosa (Figure 1). Counts were performed

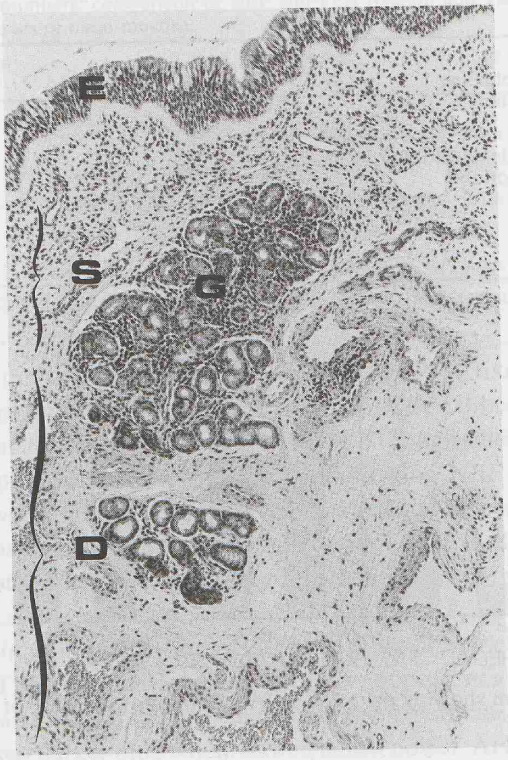

Figure 1. A typical area of nasal mucosa showing the three layers. At the top of the micrograph is the epithelium (E) supported by a thick basement membrane. The relatively thin superficial submucosa (S) is very cellular and deep to it is thicker, much less cellular deep submucosa (D) in which many spirally arranged sinusoids are seen. The centre of the field is occupied by a large group of mixed serous and mucus glands (G). PFA fixation and haematoxylin anbd eosin stain $\times 45$.

independently by two observers at $\times 400$ in each of these three areas in all specimens. The epithelial counts were made with the epithelium across the diameter of the field which measured 450 μm. For superficial submucosa counts the edge of the field was aligned along the basement membrane, and an area in which connective tissue predominated was selected. The deep submucosa counts were carried out on areas of connective tissue deep to the glandular layer. Both superficial and deep counts were done on the entire circular field of diameter 450 μm. The sum of the numbers of mast cells in the one 450 μm length of epithelium, and the numbers in one circular field each for superficial and deep tissue was then calculated to give a total mast cell number. Cell counts by the two observers correlated well and so means of their values are reported.

Mast cell counts did not conform to a normal distribution and results were,

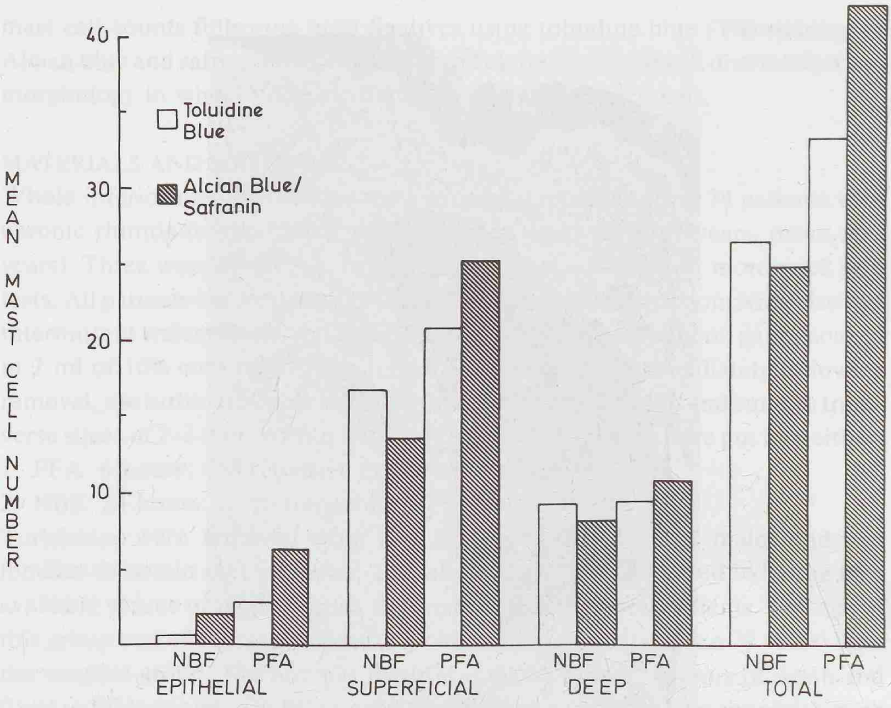

Figure 2. Histogram showing mast cell distribution following NBF or PFA fixation and TB or ABS staining.

therefore, normalised by square root transformation prior to statistical analysis. Data were analysed by paired student's t-test comparison.

RESULTS

Mast cell distribution in nasal mucosa is non-uniform (Figure 2). All combinations of fixative and stain showed small numbers of intraepithelial mast cells, intermediate numbers in deeper submucosa and by far the greatest counts in the superficial submucosa ($p < 0.001$ superficial vs. deep for PFA/ABS counts). Only one patient had no mast cells present in the epithelium. Of the remaining 13 patients, nine had 5 or more mast cells in the section of epithelium counted. Total mast cell numbers in each specimen were significantly greater following PFA fixation than NBF fixation whether stained with TB ($p < 0.05$) or ABS ($p < 0.01$), (Figure 2). Within each of the three anatomical layers, larger numbers of mast cells were also observed following PFA than NBF fixation (Table 1). The mast cell counts were significantly greater following PFA fixation with ABS staining in the epithelium, superficial submucosa and deep submucosa. Following TB staining, the difference was statistically significant only for epithelial counts.

Table 1. Mast cell numbers: comparison of NBF and PFA fixation and TB and ABS staining in different areas of nasal mucosa.

	epithelium		sup'l submucosa		deep submucosa	
	TB	ABS	TB	ABS	TB	ABS
rhinitis NBF	0.5 ± 0.9	1.9 ± 3.1	16.6 ± 5.0	13.4 ± 6.0	9.3 ± 3.3	8.0 ± 4.6
rhinitis PFA	2.7 ± 4.2*	6.4 ± 6.6***	20.7 ± 7.8	25.1 ± 13.3***	9.4 ± 4.4	10.8 ± 6.2**
autopsy PFA	0.6 ± 1.3	1.2 ± 1.6	15.4 ± 8.0	18.6 ± 14.8	8.8 ± 4.9	6.2 ± 3.6

* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ vs. NBF fixation with same staining technique.

Comparison of the two staining methods following PFA fixation showed that ABS gave better demonstration of mast cells than TB. ABS counts were higher than TB counts at all anatomical levels (Table 1), although statistically significant only for intraepithelial counts ($p < 0.001$). Following NBF fixation, staining differences between TB and ABS were less marked and less consistent. TB gave higher counts at superficial and deep submucosal sites but in almost half the NBF specimens, TB staining was very pale leading to difficulty in mast cell identification.

Using the combination of PFA and ABS, two distinct sub-types of mast cells were distinguished. The first had a bright turquoise appearance. These cells were spindle shaped and most frequently located in the superficial tissue. The second sub-type appeared darker blue when viewed at low power. At higher power these were shown to have turquoise cytoplasm with, in addition, very dark purplish-red granules. These cells were often larger, round or ovoid and more deeply located than the spindle shaped cells. Intraepithelial cells were usually very pale staining. All these cells can be shown very well by colour photography and less well in black and white.

Mast cell counts from the five autopsy subjects showed a similar distribution to those of the rhinitis patients with the greatest numbers in the superficial submucosa (Table 1). Mean mast cell counts were slightly lower at all sites, particularly superficial submucosa, although differences were not significant at present sample size. General turbinate histology was also similar in surgical and autopsy specimens.

DISCUSSION

We have demonstrated that the optimal method of preparation of nasal tissue for mast cell studies is fixation for six hours in PFA followed by ABS staining. This method also gives excellent general tissue preservation (Trotter et al., in press). Our results do not confirm the previous suggestion (Enerback et al., 1986) that mast cells are uniformly distributed in nasal mucosa. Cell numbers in the super-

ficial submucosa are significantly greater than in the deep submucosa and only small numbers are present in the epithelium. This observation is important and raises the possibility that studies based on small samples of nasal turbinate, particularly surface scrapings, may not always yield reliable findings in terms of mast cell numbers under varying conditions.

In many of the samples fixed with NBF, mast cells stained with TB are very pale and therefore difficult to count. This may in part be due to the protein "shell" formation discussed previously (Trotter et al., 1989) and such resistance to staining may be one reason why counts with this fixative tended to be lower. Longer duration in the TB stain is known to improve mast cell demonstration, but this prolongs the whole process and also leaves the background devoid of stain, so that counterstaining must then be used to allow proper observation of the tissue. ABS tended to overstain NBF fixed tissue which resulted in counting problems. With PFA fixative, the TB staining was occasionally pale, whereas ABS gave very easy mast cell identification, and revealed morphologically different sub-types.

Comparison of mean PFA mast cell counts in surgical specimens with those of the five autopsy subjects showed a similar mast cell distribution for both groups using both TB and ABS staining. The small number of epithelial mast cells in post-mortem subjects may have been due to the relatively less well preserved epithelium in these specimens which tended to be more traumatised on removal than the surgical specimens. It is possible that some of the much older autopsy group may have had "senile rhinitis", but the data suggest that turbinate mast cells are distributed non-uniformly in all subjects.

The present study confirms the previously reported heterogeneity of human mast cells (Otsuka et al., 1985; Irani et al., 1986; Gomez et al., 1987). The PFA and ABS method described here allows ready demonstration of this heterogeneity, but colour photography is needed to demonstrate this well. Although we have shown previously that the superficial layers of the submucosa have patchy distribution of mast cells, it is clear that the superficial layer as a whole has the greatest number of mast cells and contains both sub-types. Until the significance of the two sub-types is thoroughly understood and their inter-relationship established, it would seem prudent to examine whole thickness rather than biopsy or scraping samples of nasal tissue. When our PFA/ABS counts were compared with those reported by Gomez et al. (1987) using Carnoy's fixative, our decision to use PFA rather than Carnoy's is supported as our mast cell numbers are considerably higher. NBF is used as a routine fixative of pathological specimens in many laboratories. We have also shown that the total number of mast cells demonstrated in a nasal turbinate section is significantly greater following PFA than NBF fixation with either of the two staining methods used. Following PFA fixation, ABS tended to yield slightly higher mast cell counts than TB, but the

difference was significant only for epithelial counts and in the deep submucosa of autopsy specimens, number were marginally greater with TB staining. Site-dependent differences in nasal mast cell demonstration are to be anticipated in view of the apparent sub-populations of nasal mast cells which we have demonstrated, but for studies requiring a single reliable method of nasal mast cell demonstration with good tissue preservation the present results support the selection of PFA fixation followed by ABS staining.

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