

Identification of mast cells in bronchoalveolar lavage fluid

Comparison between different fixation and staining methods.

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The present study was carried out to compare the effectiveness of different fixation and staining methods in the identification of mast cells obtained by bronchoalveolar lavage from patients with interstitial lung diseases. Cell preparations were fixed with formaldehyde or methanol. Mast cells were identified by metachromatic staining with May Grünwald Giemsa, Toluidine blue or Gallamine blue Giemsa. After formaldehyde fixation only a few mast cells were identified, regardless of the stain method used. In contrast, a significantly higher number of mast cells were observed after methanol fixation. Using this fixative, Toluidine blue stain showed a higher number of mast cells than May Grünwald Giemsa. However, there was no difference between Toluidine and Gallamine blue Giemsa in the number of cells observed. The easy identification of mast cells after staining with toluidine, combined with its easy application, suggest that Toluidine blue stain after methanol fixation is the most useful method for determining the presence of mast cells in lavage fluid.

Key words: bronchoalveolar lavage; fixation methods; mast cells; staining methods.

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CLINICAL ASPECTS

Increased numbers of mast cells have been found in the bronchoalveolar lavage fluid of patients with lung diseases. However, different conditions of fixation and staining may be required for their identification. Toluidine blue stain after methanol fixation is more useful than Gallamine blue Giemsa and May Grünwald Giemsa stains in determining the presence of mast cells in lavage fluid.

Mast cells are widely distributed throughout the respiratory tract, both in the airways and in the alveoli. In the airways, mast cells are located beneath the respiratory epithelium superficial to the basement membrane and between the epithelial cells. Mast cells are also found in the alveolar walls where they constitute almost 2% of the surface area (12,14). Thus it is not surprising that mast cells are identified in bronchoalveolar lavage (BAL) fluid. Increased numbers of mast cells have been found in BAL fluid of

several interstitial lung diseases, mainly extrinsic allergic alveolitis (13). It is now clear that different conditions of fixation and staining may be required to accurately identify the mast cells in lavage fluid. In this study we have compared the effectiveness of different fixation and staining methods in the identification of mast cells in BAL from patients with interstitial lung diseases. The fixatives used were methanol and a formol saline solution; the stain solutions were May Grünwald Giemsa, Toluidine blue and

Gallamine blue Giemsa. We selected these stains because May Grünwald Giemsa is widely used in BAL cytological studies, and Toluidine blue as well as Gallamine blue Giemsa are especially effective in staining the mast cell granules. Gallamine blue Giemsa, however, has never been used in the identification of BAL mast cells.

METHODS

We studied 20 patients who underwent BAL as part of the investigation of interstitial lung disease. The histologic diagnoses were: idiopathic pulmonary fibrosis, 9 cases; sarcoidosis, 4 cases; pulmonary fibrosis associated with collagen vascular diseases, 3 cases (rheumatoid arthritis, systemic sclerosis, dermatomyositis); pulmonary asbestosis, 2 cases, and extrinsic allergic alveolitis and histiocytosis X, 1 case. Diagnoses were obtained according to established criteria (6). Idiopathic pulmonary fibrosis and histiocytosis X were diagnosed by open lung biopsy. The four patients with sarcoidosis had non-caseating granulomas in transbronchial lung biopsy.

Bronchoalveolar lavage was performed via an Olympus fiberoptic bronchoscope as previously reported (23). The tip of the bronchoscope was wedged into either the lingula or the middle lobe and 150 ml of sterile 0.9% saline solution were introduced in 50 ml aliquots and gently aspirated. The fluid obtained after the introduction of the first 50 ml aliquot was discarded. The rest of the fluid was collected into a sterile siliconized glass container and immediately analysed. The total cell count was calculated in Neubauer's camera. The liquid was then centrifuged (500g, 10 min) and the cell pellet resuspended in phosphate buffer solution (PBS). Cell preparations were made by slow-speed cytocentrifugation (500g, 10 min) (Cytospin 2, Shandon) using $10\text{--}20 \times 10^4$ cells and fixed and stained as described below. Cell differentials were obtained after counting 400 cells in slides fixed with methanol and stained by May Grünwald Giemsa. The percentage of macrophages, lymphocytes, eosinophils and neutrophils was evaluated.

Cell preparations were fixed according to two methods: cell smears were incubated in a

formaldehyde-saline solution (10% formaldehyde in 0.9 saline, 1:10) for 30 min or in absolute methanol for 15 min. Both fixations were conducted at room temperature.

Smears fixed in both fixatives were then stained according to three methods: May Grünwald Giemsa, Toluidine blue and Gallamine blue Giemsa. In the first case, slides were incubated in undiluted May Grünwald (Merck 1424) for 1 min, May Grünwald diluted 1:2 in distilled water for 1 min and Giemsa (Merck 9204) diluted 1:10 in distilled water for 5 min. With Toluidine blue (Merck 15930), cell smears were incubated in a Toluidine blue solution for 45 min (pH 5). This solution was prepared by dissolving 400 μ l of acetic glacial acid and 0.025 g of Toluidine blue in 30 ml of absolute ethanol, adding distilled water to make a total volume of 100 ml. Finally, with the Gallamine-blue Giemsa stain, cell preparations were incubated in Gallamine-blue stain for 16 h at room temperature. Afterward, the slides were washed with distilled water for 10 min. After washing, the cell preparations were incubated in Giemsa stain (pH 6.7) at 40°C for 60 min and in Giemsa stain (pH 6.6) at 40°C for 40 min. The slides were then washed again with distilled water. Gallamine-blue stain was prepared by dissolving 5 g of aluminum potassium sulfate (Merck 1047) in 0.1 g of Gallamine blue (Difco 8236.11) and then adding distilled water to make a total volume of 100 ml. The resulting solution was then heated until ebullition and filtered. This type of solution is stable for 4 weeks at room temperature. Mast cells were identified by the presence of characteristic methachromatic granules and counted in 20 consecutive fields with $\times 1000$ magnification in a light microscope.

Statistical analysis

Values are presented as the mean $\pm$ SD. Comparison between the fixation and stain methods was evaluated in accordance with the Wilcoxon's rank sum test. A $P < 0.05$ was considered as significant.

RESULTS

Bronchoalveolar lavage was carried out without difficulty or complications. The endoscopic examination of the bronchial tree was normal in all subjects with no evidence of inflammation or purulent secretions. The mean values and ranges for lavage fluid recovered and absolute and differential cell counts are shown in Table 1.

Mast cells were observed in 18 of 20 cases. After methanol fixation, mast cells were recognized in 18 cases, and after formol fixation, in only 12. In slides stained with May Grünwald Giemsa, it was easy to identify the mast cells because of their prominent purple granules. Using Gallamine blue Giemsa stain, mast cells were also recognized without difficulty. With this staining method the cytoplasmatic granules showed a strong, red stain. In contrast, eosinophilic granules were stained yellow and other nucleated cells appeared blue. After staining with Toluidine blue, mast cells were also easily identified since the cytoplasmatic granules were stained violet red, contrasting with the pale-blue staining of other nucleated cells.

After formaldehyde fixation, only a few cells fulfilled our criteria for mast cells, regardless of the staining method employed. Using May Grünwald Giemsa, 0.2 ± 0.89 (range 0-4) mast cells were recognized (Table 2.). After staining with Gallamine blue Giemsa, we observed 1.6 ± 1.9 (range 0-6) cells. With Toluidine blue stain, 0.2 ± 0.5 cells (range 0-2) were identified. In contrast, a significantly higher number of mast cells were observed after methanol fixa-

tion. When May Grünwald Giemsa stain was used, 1.8 ± 2.8 (range 0-11) mast cells were identified ($P < 0.05$, with respect to formaldehyde fixation). After Gallamine blue Giemsa stain we found 3.3 ± 5.8 (range 0-26) cells ($P < 0.05$), and after Toluidine blue stain, 4.6 ± 7.7 cells (range 0-29) ($P < 0.01$). In addition, the Toluidine blue stain showed a significantly higher number of mast cells than May Grünwald Giemsa stain when methanol fixation was used ($P < 0.05$). In contrast, there was no significant difference between Gallamine blue Giemsa and Toluidine blue stains after methanol fixation (4.6 ± 7.7 vs 3.3 ± 5.8 cells, respectively).

DISCUSSION

Mast cells are thought to play an important role in the pathogenesis of several interstitial lung diseases, such as allergic extrinsic alveolitis, idiopathic pulmonary fibrosis and sarcoidosis. Patterson et al. (18) described for the first time the presence of methachromatic cells in bronchial lavage. The cells were called basophiloid because they could represent either mast cells or basophils. Nowadays, it is accepted that methachromatic cells in BAL have the nuclear and granular configuration of mast cells (22). In comparison with other cells recovered by BAL, it seems reasonable to assume that mast cells originate from both the alveolar and the small airways adjacent to the alveoli. However, the identification of mast cells has long been a problem. Michels (16) stated in 1938, that much misleading research resulted from the use of

Table 1.

Disease	Cases	Lavage fluid recovered, total and differential cell counts					
		BAL fluid recovered (ml)	Total cells ($\times 10^3/\text{ml}$)	Macrophages	Lymphocytes	Neutrophils	Eosinophils
IPF	9	75 ± 18	7.5 ± 4.1	$90 \pm 8^*$	$5 \pm 6^*$	$6 \pm 1^*$	$1 \pm 0.8^*$
IPF-CVD	3	55 ± 15	8.4 ± 0.6	87 ± 8	4 ± 4	6 ± 4	2 ± 2
Sarcoidosis	4	82 ± 6	5.8 ± 1.3	61 ± 31	37 ± 31	0.1 ± 0.2	1 ± 1
Asbestosis	2	91 ± 13	18 ± 10	90 ± 7	3 ± 2	2 ± 1	4 ± 4
EAA	1	42	8.0	77	23	0	0
HX	1	84	9.2	96	4	0	0

IPF: idiopathic pulmonary fibrosis; CVD: collagen vascular disease; EAA: extrinsic allergic alveolitis; HX: histiocytosis X;

*: percentage of cells.

such fixatives as 10% – 40% aqueous formalin solutions. Also Lempert et al. (15) found that sections of human myocardium fixed in aqueous formalin yielded lower mast cell counts than corresponding sections fixed in alcoholic formalin. Asboe-Hansen & Zachariae (2) strongly favoured fixation in 4% lead subacetate solution for the same reasons. The variability in mast cell count found by different investigators has been related to differences in BAL technique and the different fixatives and stains used in their identification. In this context, Flint et al. have demonstrated that filtering the lavage fluid through gauze to remove mucus could decrease the percentage of mast cells (8). The different affinity of mast cells to fixation and stain solutions has been related to the existence of two subtypes of these cells: the mucosal mast cell and the connective mast cell (7).

In this study, we have compared the effectiveness of different fixatives and stains in the identification of mast cells. We used a formol saline solution and methanol as fixatives, as well as three different stains. May Grünwald Giemsa is widely used in the BAL differential counts (13, 20, 23) and Toluidine as well as Gallamine blue Giemsa methachromatically stain the mast cell granules (1, 3, 4). This methachromasia arises from a shift in the colour spectrum of the dye following its interaction with the proteoglycans contained in the granules (19). Although Gallamine blue Giemsa stain has been used in the identification of bone marrow mast cells, to our knowledge it has never been employed in the identification of lavage fluid mast cells (4, 5). Bone marrow mast cells are similar to those found in biopsy material obtained from bronchial mucosa and lungs; they contain methachromatic-positive granules, although they have some differences

in their size, shape, as well as in the distribution and structure of cytoplasmatic granules with respect to lung mast cells (22).

Our results revealed that after formaldehyde fixation, few cells took up the stains. In contrast, several mast cells were stained after methanol fixation. These findings are in accord with previous studies in which similar results were observed (9, 13, 17). Other formol-free fixatives used in these investigations were the Carnoy's solution and the Mota's fluid. It has been reported, however, that mast cell counts obtained employing Carnoy's fixative are similar to those obtained after methanol (1, 13). In addition, it appears that Carnoy's solution is less satisfactory as a fixative than methanol because it can cause dissolution of some mast cell granules. Furthermore, some macrophages contain material which may be heavily stained after exposure to Toluidine blue when Carnoy's solution is used (13). Our findings indicate, as previously reported, that mast cells found in interstitial lung diseases resemble mucosal mast cells in their failure to stain after fixation in formol fixatives (9, 13, 17).

According to the results obtained in this study, Toluidine blue and Gallamine blue Giemsa after fixation with methanol are the most useful methods for mast cell identification. Toluidine blue only faintly stained the other nuclear cells observed in BAL cell preparations, and moreover, no staining of extracellular material was observed. In a similar study, Haslam et al. (13) noted that macrophages can contain heavily stained material after exposure to Toluidine blue stain and formol-free fixatives; this was not the case, however, in our study. This difference could be explained by the different methodology used, including the incubation time and the type of Toluidine employed. Gallamine blue Giemsa stain yielded similar results to those obtained after Toluidine exposure, although it stained other nucleated cells as well, making it more difficult to recognize the mast cells. As the preparation of Gallamine blue Giemsa stain is more difficult and takes more time to use than the Toluidine technique, we think that Toluidine staining is the most useful method for identifying mast cells in BAL.

Table 2.

Mast cell counts in bronchoalveolar lavage fluid

Staining method	Formaldehyde fixation	Methanol fixation
May Grünwald Giemsa	0.2±0.89*	1.8±2.8
Gallamine blue Giemsa	1.6±1.9	3.3±5.8
Toluidine blue	0.2±0.5	4.6±7.7

* Number of mast cells in 20 microscopic fields

Other stain techniques have also been used in the identification of mast cells. The Alcian blue stain allows for easy mast cell identification (9); however, mucus can also take up this stain and when it is attached to other cells, resembles mast cells. In addition, macrophages occasionally stain with Alcian blue, especially when the incubation time is long, resulting in an overcounting of mast cells. Safranin O solution has also been used as counterstaining after Alcian blue and it has been demonstrated to be a useful method for identifying connective mast cells (10, 11). The Alcian blue - Safranin O method, however, has not been demonstrated as better than Toluidine in the identification of mast cells (21). Finally, barberine sulfate is a fluorescent cationic dye which binds with mast cell granules (11) but, the fluorescence fades in a few hours and, consequently, the preparations are not permanent (17).

In most of our cases, less than five mast cells were identified after examining 20 microscope fields. These findings suggest that the conventional BAL differential count through the screening of 400-500 cells is too low for a reliable count of mast cells. The number of BAL mast cells should be evaluated by examining a higher number of cells per slide.

In summary, our study reveals that staining with Toluidine blue or Gallamine blue Giemsa solutions after methanol fixation, show similar results in identifying mast cells in lavage fluid. However, the easier identification of these cells after staining with Toluidine, combined with its easy application, appears to indicate that Toluidine blue stain after methanol fixation is the most useful technique for evaluating the presence of bronchoalveolar lavage mast cells.

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