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Alcian Blue and Pyronine Y histochemical stains permit assessment of multiple parameters in pulmonary disease models

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Abstract

Utilization of a combined Alcian Blue and Pyronine Y histochemical method for the assessment of multiple parameters in the respiratory tract of various species is described. Acidic mucins were deep blue (sialylated mucins), red (sulfated mucins), or variably purple (mixture of sialylated/sulfated mucins), and differential mucus production was readily detected in a murine respiratory syncytial virus vaccine model of pulmonary inflammation. Elastic fibers stained red in the walls of pulmonary arteries, connecting airways, alveolar septa, and subpleural interstitium. Mast cells had red to red-purple granular cytoplasmic staining. Nuclei were ubiquitously counterstained pale blue. Representative staining was detected in tissues from multiple species including inbred mice, rats, ferrets, cats, dogs, sheep, and pigs. The fluorescent property of the stained tissues offers additional modalities with which to analyze tissue sections. This histochemical technique detects multiple critical parameters in routine paraffin sections of lung tissue, reduces the need for repeated serial sectioning and staining, and is cost-effective and simple to perform.

Keywords

Alcian Blue; Elastic fibers; Lung; Mast cells; Mucin; Mucus; Pyronine Y; Respiratory Syncytial Virus

Mucus expression, elastic fiber integrity, and mast cell numbers are important morphologic parameters that are used to gauge chronic pulmonary disease progression and severity.^{1,3,7} Assessment of these and other parameters is essential to properly characterize and compare animals in experimental and control treatment groups. Individual histochemical stains are often selectively utilized to detect specific structures (e.g. mast cell granules). A staining method that labels multiple tissue elements in a single section would save time and resources and enable assessment of possible topographic relationships between these elements. Here we describe the application of Alcian Blue and Pyronine Y (AB/PY) to simultaneously assess multiple parameters in pulmonary tissues.

Animals used in the study were approved by the University of Iowa Institutional Animal Care and Use Committee. Formalin-fixed, paraffin-embedded necropsy tissues (one tissue section per animal and stain) of multiple species were collected from archival blocks. Whereas the lungs of mice were inflated with fixative (10% neutral buffered formalin) as a routine necropsy

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procedure, the postmortem processing of lung tissue from other species beyond “formalin fixation” was not determined. Differential mucus production was assessed in a proven vaccine-induced respiratory syncytial virus (RSV) inflammation model.⁴ Lung sections were examined by a pathologist blinded to the study groups, and scoring parameters were applied as described below.

For the AB/PY method, Iron Pyronine Y solution (a mixture of 300 mL of 0.5% aqueous Pyronine Y, Cat# U724-03, J T Baker Chemical Company, Phillipsburg, NJ, USA and 20 mL of ferric chloride, #I88, Fisher, Pittsburgh, PA) at pH 2.5 was applied for 6 h at room temperature. Next the slides were rinsed in distilled water, immersed into a 3% acetic acid solution for 3 min followed by Alcian Blue solution (pH 2.5) (Sigma Aldrich, St. Louis, MO, USA) for 30 min. For the Periodic Acid Schiff (PAS) method (Sigma Aldrich, St. Louis, MO, USA), serial slides were immersed in 0.5% periodic acid for 10 min, rinsed with distilled water, and placed in Schiff’s solution for 15 min. The respective slides were rinsed in running tap water for 10 min, counterstained with Harris Hematoxylin (1 min), rinsed, blued in ammonia water, dehydrated, cleared, and mounted with cover slips. For fluorescence, the AB/PY stained sections were examined under a fluorescent microscope (Eclipse E600, Nikon) with a Tritc filter (Exciter-540/25 DM-565, BA 605/55), and the resulting digital image was transformed from grayscale to green to represent the fluorescence.

In AB/PY-stained normal lung from inbred mice, mucus had a deep blue to red-purple color and was seen within surface epithelial goblet cells and submucosal gland epithelium; both were seen only in the very proximal trachea consistent with their normal distribution in the mouse (Fig. 1). The tracheal cartilage was highlighted red to red purple. Elastic fibers stained red and were easily detected because they effectively contrasted with the pale blue nuclear background of the adjacent tissue. As expected, the elastic fiber staining was detected in vascular walls (i.e. pulmonary arteries with decreased staining in smaller-sized vessels), walls of connecting airways, variably present in alveolar septal walls, and subjacent to the visceral pleura. Mast cells were readily detected due to red or less commonly red-purple staining of cytoplasmic granules and were often located adjacent to larger airways (Fig. 2).

In the murine RSV inflammation model, the difference in mucus production was readily distinguished, even at low magnification, making tissue assessment relatively straightforward for the pathologist. RSV G protein-primed mice infected with RSV, but not beta-galactosidase-primed (control) mice, had prominent deep blue granular staining of the apical or entire cytoplasm of epithelial cells in larger connecting airways (Figs. 3, 4). Deep blue-stained epithelium in G-primed mice also contained rare small red-purple punctate foci not seen in controls.

Findings in AB/PY-stained lung tissues from multiple species commonly used in experimental disease models were similar to those observed in the mouse with only mild interspecies variation (Table 1). For instance, mice do not have detectable goblet cells or submucosal glands in distal airways as do pig or ferret, and these species differences in cellular distribution of mucus were easily distinguished in AB/PY-stained sections. Furthermore, the AB/PY stain distinguished qualitative differences in mucus production, including a significant amount of sulfated mucins in pig airways compared to mice. PAS and AB/PY methods for detection of neutral and acidic mucins, respectively, revealed a remarkably similar cellular distribution of mucus as detected by each method in both the mouse and pig lung (Figs. 5, 6). Species such as the dog and sheep had comparatively less elastic fiber staining in the walls of airways and in the alveolar septa, which was confirmed with another elastic fiber stain (iron gallein elastic, data not shown). AB/PY staining of normal cat lung detected red elastic fibers subjacent to airway epithelium, whereas tissue from a chronic bronchitis/bronchiectasis model lacked subepithelial elastic fiber staining (Figs. 7, 8). As in mice, mast cells were easily detected in

species that have resident pulmonary mast cells (e.g., rat and cat). Similar granular cytoplasmic staining was detected in a dermal mast cell tumor from a dog (data not shown).

Finally, we examined tissue sections stained with AB/PY for putative fluorescence. Mast cell granules and elastic fibers/lamina of arteries, as seen in light microscopy, were readily detectable and fluorescent in AB/PY-stained sections (Fig. 9). Moderate background fluorescence in the tissue was more extensive in distribution than anticipated from parallel assessment of the same section using light microscopy.

Alcian Blue has historically been used to detect acidic mucus, and pretreatment with sialidase or manipulation of the pH have been used to further define sialidase-resistant sialomucins or sulfated mucins.^{11,12} This is in contrast with another commonly used histochemical method, the PAS technique, which detects neutral mucins.¹¹ Pyronine Y, an iron-based method, selectively stains sulfated mucins in addition to aiding detection of other tissue parameters including RNA, elastic fibers, and mast cell granules.^{2,8,14} While the AB/PY histochemical method has been reported,² its application to experimental models of pulmonary disease as a broad spectrum tissue marker is not well described.

The use of AB/PY allows for discriminatory detection of cellular acidic mucus alterations through recognition of sialylated (deep blue staining), sialylated/sulfated (variable purple staining), or sulfated (red staining) mucus.¹⁰ Diseases such as cystic fibrosis or chronic bronchitis can alter production of sialylated and/or sulfated mucin.⁵ Furthermore, alpha 2,3-sialyltransferase activity, an enzyme responsible for sialylation of mucus, is increased by expression of tumor necrosis factor- α , thus increasing the proportion of sialylated acidic mucus produced.⁶ Both PAS and AB/PY methods showed similar cellular tissue distribution of mucus in the lung and suggests that AB/PY could be a more informative method than PAS to screen for cellular mucus alterations; however, it cannot replace PAS for neutral mucus detection.

In addition to mucus, AB/PY detected elastic fibers and mast cells, both important parameters in assessment of pulmonary disease.^{3,8} Whether AB/PY detects all mast cells or only certain mast cell subpopulations in the species examined here has not been determined, since various factors including genotype and fixation may affect mast cell detection.¹⁵ The fluorescent property of AB/PY-stained sections permits a single paraffin section to be examined by both light and fluorescence microscopy. RNase pretreatment of tissues is reported to significantly mitigate background fluorescence.¹³

Because the AB/PY method detects multiple parameters that may be altered in lung disease, serial sections for additional stains are reduced. In our laboratory, the cost comparison (materials and labor) between AB/PY and representative alternative methods for assessment of three parameters such as mucus (e.g. PAS), elastin (e.g. iron gallein elastic), and mast cells (e.g. Giemsa) indicate the AB/PY stain provides a cost savings of approximately 74%. Furthermore, the simple addition of Pyronine Y to an established Alcian Blue protocol increases the cost by a mere 5%, suggesting that the AB/PY stain is a cost-effective “upgrade” from standard Alcian Blue protocols.

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Figure 1.

Fig. 1. Proximal trachea; BALB/cNCr mouse. The tracheal cartilage stains red with mucus stained variably blue to red-purple in proximal tracheal submucosal glands (arrows). Alcian Blue/Pyronine Y. Bar = 0.5 mm.

Fig. 2. Lung; BALB/cNCr mouse. Peribronchial mast cells (arrows) have red granular cytoplasmic staining. Alcian Blue/Pyronine Y. Bar = 50 μ m.

Fig. 3. Lung; BALB/cNCr mouse. Airway from a beta-galactosidase-primed RSV infected mouse with little to no blue staining of airway epithelium. Alcian Blue/Pyronine Y. Bar = 100 μ m.

Fig. 4. Lung; BALB/cNCr mouse. Airway from a RSV G-protein-primed RSV-infected mouse. Note the intense deep blue mucus detection (arrows) in connecting airway epithelium. Alcian Blue/Pyronine Y. Bar = 100 μ m.

Fig. 5. Lung; Pig. Normal bronchus with submucosal gland and surface epithelial mucus staining magenta. Periodic Acid Schiff (PAS). Bar = 50 μ m.

Fig. 6. Lung; Pig, serial section of Figure 5. Overall staining pattern is similar to Figure 5 (PAS), but sialylated (blue), sulfated (red) or mixed (purple) mucus can be differentiated. Alcian Blue/Pyronine Y. Bar = 50 μ m.

Fig. 7. Lung; Cat. Arrows indicate elastic fibers subjacent to bronchial epithelium. Bar = 50 μ m.

Fig. 8. Lung; Cat. Chronic bronchitis with severe bronchiectasis demonstrating qualitative change in mucus expression (increased red staining) and a lack of elastic fibers staining. Alcian Blue/Pyronine Y. Bar = 50 μ m.

Fig. 9. Artery; BALB/cN mouse. Elastic fibers stain red, Alcian Blue/Pyronine Y. Inset, Intense fluorescence of elastic fibers in the same section examined via fluorescent microscopy. Bar = Bar = 33 μ m.

Summary of Alcian Blue and Pyronine Y staining of mucus, elastic fibers, and mast cells in multiple experimental model species. Stain intensity of select parameters were characterized as moderate to strong staining (++), detectable staining (+), or not assessable (N/A).

Table 1

<u>Species</u> (number of animals)	<u>Mucus</u>		<u>Elastic fibers</u>		<u>Mast cells</u>	
	Blue	Red/Purple-red	Perivascular	Airway	Interstitial	
Mouse (n=9)	++	+	++	++	++	++
Rat (n=2)	++	+	++	++	++	++
Ferret (n=3)	++	++	++	++	++	N/A
Rabbit (n=2)	++	++	++	++	++	N/A
Cat (n=3)	++	++	++	++	++	++
Dog (n=3)	+	++	++	+	+	++
Sheep (n=2)	+	++	++	+	+	N/A
Pig (n=3)	++	++	++	++	++	N/A