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Identification of Mast Cells and Mast Cell Subpopulations

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Abstract

Mast cells generate mediators of inflammation which are stored in granules and secreted on activation either by allergen crosslinking of membrane-bound IgE or through other stimuli. Most methods for mast cell identification rely on the histochemical detection of constituents of the secretory granules. Although staining for mast cells with histochemical stains can be rapid and relatively inexpensive, it is not always possible to distinguish reliably between mast cells and basophils in tissues. A further problem with the staining of mast cells with commonly used basic dyes is that the reagents employed to fix the tissues can influence the results, leading to confusion regarding the numbers of mast cells present in various tissues. Recognition that there is considerable heterogeneity between mast cell populations in the degree to which staining properties are lost with formalin fixation has led to mast cell subsets being defined on this basis.

The development and application of procedures for identifying mast cell proteases has led to important advances in our understanding of the role of mast cells and in the nature of heterogeneity in man. The techniques described here should allow the reliable detection of mast cells and mast cell subsets in a range of tissues and cell preparations. There will be a continuing need for validation, for consideration of potential sources of error, and for the development of new and more reliable techniques for mast cell identification.

Key Words: Mast cell; basophil; protease; chymase; Alcian blue.

1. Introduction

Mast cells were originally described by Ehrlich in 1879 as granular cells staining metachromatically with a basic dye ***(1)***. Thinking that the granules had been phagocytosed, he named them "mast zellen" (well-fed cells). It has since become clear that the granules contain mediators of inflammation which are generated within the mast cells and which are secreted following cell activation,

From: *Methods in Molecular Medicine: Allergy Methods and Protocols*
Edited by: M. G. Jones and P. Lympany © Humana Press Inc., Totowa, NJ

either induced by allergen crosslinking membrane-bound IgE or through a range of other stimuli. Certain mediators (including heparin and a trypsin-like enzyme named tryptase) are stored in the granules of all mast cells, and have been used as markers for mast cells in cell preparations and tissues, as have IgE and the membrane component c-*kit*. Other unique mast cell products such as chymase and mast cell carboxypeptidase are present only in a subset of these cells and have become markers for mast cell heterogeneity ***(2,3)***.

For over a hundred years following their discovery, mast cells continued to be identified using basic dyes. The dyes most commonly used today are Alcian blue and toluidine blue. At neutral pH both these dyes bind to a variety of tissue components. However, under the highly acidic conditions at which they are employed for the identification of mast cells, only the highly sulfated proteoglycans remain positively charged and therefore capable of binding these basic dyes. Binding of toluidine blue to the repetitively charged side chains of heparin in the mast cell granule brings the colored ionic portions of the dye into close alignment, causing a shift in the wavelength of light absorbed. This color shift is termed metachromasia and is seen for toluidine blue but not for Alcian blue. Staining for mast cells with histochemical stains can be rapid, and the dyes are relatively inexpensive. However, these reagents do not provide means for distinguishing reliably between mast cells and basophils in tissues as the highly charged proteoglycans of basophils may also bind them.

A further problem with the staining of mast cells with basic dyes is that the reagents employed to fix the tissues can influence the results. Fixation in the most commonly used formaldehyde preparations can lead to the loss of mast cell staining with toluidine blue or Alcian blue. Until the late 1960s, the extent of this problem was not appreciated, leading to confusion regarding the numbers of mast cells present in various tissues.

Recognition that there is considerable heterogeneity between mast cell populations in the degree to which staining properties are lost within formalin fixation has led to mast cell subsets being defined on this basis. Mucosal tissues of humans and rodents readily lose their ability to stain with basic dyes following formaldehyde fixation (as can be seen when compared with findings from certain other fixatives such as Carnoy's fluid). The staining of mast cells in connective tissue on the other hand is relatively little affected by formaldehyde fixation. Staining properties of mast cells may be altered by disease; for example, selective increases in the proportions of the formaldehyde-resistant mast cells have been noted in bronchoalveolar lavage fluid from patients with certain fibrotic lung conditions ***(4)***.

The ability of mast cells in formaldehyde-fixed tissues to be stained may be restored by prolonging the staining period to several days or by treating tissue sections with trypsin ***(4,5)***. This suggests that formaldehyde may induce

crosslinking of the protein shell around the proteoglycan and thus restrict dye binding. In this case, the proteinaceous content of the mast cell secretory granule as well as the proteoglycan may contribute to the dye-binding properties of mast cells. With toluidine and Alcian blue dyes, it is important for mast cell specificity that the staining is performed at very low pH values (e.g., pH 0.5). Even so, with prolonged staining periods, it may be difficult to distinguish mast cells from other cell types on the basis of staining alone. With a staining period greater than 6 h, metachromatic staining of eosinophils has been observed with toluidine blue and after 24 h, erythrocytes and some lymphocytes become intensely stained ***(4)***. Careful standardization of fixation and staining procedures is crucial.

Toluidine and Alcian blue have come to be the most widely employed histological stains for mast cells in fixed tissues or cell preparations, and protocols for their use are provided here. Differences in performance between these two dyes have been reported but not consistently, perhaps because of variation between individual batches. A staining procedure developed by Kimura and colleagues ***(6)*** also involves staining with toluidine blue and a protocol for this is also provided.

There are numerous other procedures in which the detection of mast cells relies on principles similar to those involved with Alcian or toluidine blue. Other basic dyes include safranin O, astra blue, azure A and B, and thionine. Safranin, a red stain which resembles toluidine blue in chemical structure and which stains mast cells orange, has been used in a staining procedure either in sequence or together with Alcian blue (reviewed in **Ref.** ***[7]***). Differential staining of mast cell granules with Alcian blue and safranin has been observed in human and rodent tissues, which may be related to the degree of sulfation of proteoglycans. Other approaches employed to detect mast cells have relied on the binding of proteoglycans of fluorescent dyes such as berberine sulfate or acridine orange or avidin conjugated to enzymes or fluorescent compounds.

Constituents of the mast cell secretory granule other than proteoglycans may offer advantages as markers for this cell type. Some success has been reported using an immunohistochemical procedure with an antibody specific to histamine ***(8)***. However, the advent of well-characterized monoclonal antibodies specific for some of the mast cell proteases has led to immunohistochemical procedures with specific antibodies becoming the method of choice for the detection of mast cells in human tissues. The tryptic serine protease tryptase appears to be almost unique to mature mast cells and as such is an excellent maker for this cell type. Immunocytochemical procedures allow selective targeting of this major granule constituent, and employing secondary antibody and enzyme conjugate amplification procedures allows higher signal to noise ratios to be achieved than with conventional basic dye procedures ***(9)***. Chymase, a chymotryptic serine

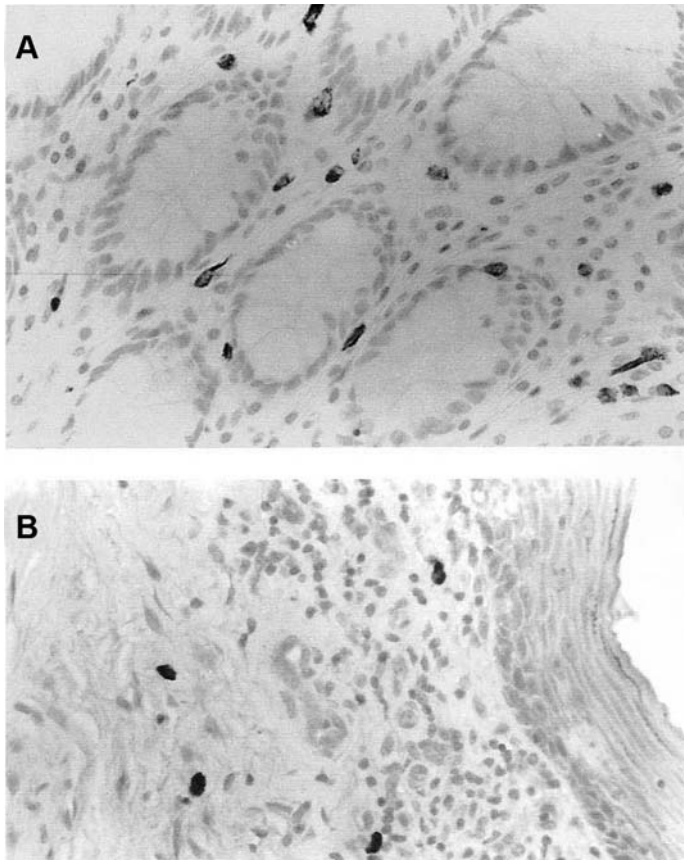

Fig. 1. Mast cells identified **(A)** around the crypts in human colonic tissue, using an immunohistochemical procedure specific for tryptase (AA1) and **(B)** in the dermis of skin using an antibody specific for chymase (CC1).

protease, is unique to a subpopulation of mast cells which predominates in normal connective tissue sites and as such has become an important marker for mast cell heterogeneity in human mast cell populations. The identification of mast cells with antibodies specific for tryptase or chymase is illustrated in **Fig. 1**. On the basis of double-labeling procedures in immunohistochemistry, mast cells can be categorized according to the presence of both tryptase and chymase (MC_{TC}) or of tryptase but not chymase (MC_T) ***(2,10)***.

Selective depletion of the (MC_T) population has been noted in the gut of AIDS patients and a selective expansion in the affected tissues in a range of conditions involving inflammation or tissue remodeling including rhinitis,

conjunctivitis, scleroderma, rheumatoid arthritis, and osteoarthritis (reviewed in **Ref.** ***[11]***). Available evidence suggests that certain other mast cell proteases including a carboxypeptidase and cathepsin G are localized preferentially in the MC_{TC} subset, and certain inflammatory cytokines may be selectively present in each of the major subsets defined, although this has not been investigated systematically with a range of tissues.

Sensitive immunocytochemical procedures for mast cell proteases have now largely supplanted earlier approaches involving the application of chromogenic substrates for histochemical detection of these enzymes. Immunocytochemistry probably offers a greater chance for standardization, and antigenicity may be better conserved than enzymatic activity during storage and processing of tissues. Nevertheless, the relative proportions of MC_T and MC_{TC} reported have varied between studies, even when results with nondispersed tissues have been compared. The use of different antibodies and different staining protocols has undoubtedly contributed to this, and there is a constant need for optimization and standardization. When double-labeling procedures are employed for tryptase and chymase, it is essential that there is effective detection of both these proteases. Variations in periods of incubation of primary antibodies on tissue sections can result in major differences in the proportions of MC_T and MC_{TC} subpopulations detected and even in the appearance of cells with chymase but not tryptase MC_c ***(12)***.

The development and application of procedures for identifying mast cell proteases has led to important advances in our understanding of the role of mast cells and in the nature of heterogeneity in man. With nonhuman species, suitable antibodies which could allow similar approaches to be pursued are not generally available. Moreover, there are major interspecies differences in the types and distributions of proteases present in mast cells, and simple categorization of MC_T and MC_{TC} populations will in most cases be inappropriate. Thus, for example, multiple chymases (with quite different patterns of expression) have been characterized in several species of small mammal, but just one in man (reviewed in **Ref.** ***[11]***). It also seems likely that several subsets of human mast cell will be defined using immunohistochemical procedures with antibodies specific for proteases or other mast cell components.

An unresolved issue is the extent to which basophils may be detected as mast cells using the immunocytochemical techniques currently available. The early studies suggested that the quantities of tryptase or chymase present in basophils were too small to be detected. However, a report that substantial quantities of tryptase or chymase may be present in basophilic cells in peripheral blood from certain allergic subjects ***(13)*** calls for further evaluation of the potential for basophil staining. The advent of antibodies specific for unique constituents of the human basophil ***(14)*** should allow this point to be addressed.

Most methods for identification of mast cells rely on the detection of constituents of secretory granules. In most cases, mast cell degranulation may be partial rather than total, but there may be particular difficulties in detecting mast cells where a substantial degree of degranulation has occurred. The potential for "phantom mast cells" which fail to stain with basic dyes is well established ***(15)*** and it seems likely that even with the more sensitive techniques, numbers of mast cells may sometimes be underestimated. To date, the only marker selective for mast cells which is not granule-associated is c-*kit*. Immunochemistry with the c-*kit*-specific antibody YB5.B8 has been used for the identification of mast cells ***(16)***, but it has proved unsuitable for paraffin-embedded tissues.

The techniques described here should allow the reliable detection of mast cells and mast cell subsets in a range of tissues and cell preparations. There will be a continuing need for validation, for consideration of potential sources of error, and for the development of new and more reliable techniques for mast cell identification.

2. Materials

2.1. Fixatives

1. Neutral buffered formalin: 100 mL 40% formaldehyde solution, 3.48 g NaH_2PO_4, 6.5 g Na_2HPO_4, 900 mL distilled water.
2. Carnoy's fixative: 60% ethanol, 30% chloroform, 10% acetic acid. The chloroform in this solution can corrode certain plastics, so it should be prepared and stored in glassware.

2.2. Histochemistry with Embedded Tissues

1. Histoclear II histological clearing agent (Raymond Lamb, Eastbourne). This is a less toxic alternative to xylene.
2. Alcian blue: 0.5% Alcian blue 8GX (certified stain CI 74240) in 0.5 M HCl (*see* **Note 1**).
3. Toluidine blue: 0.5% toluidine blue (certified stain CI 52040) in 0.5 M HCl. Add the stain to the acid gradually, with continual magnetic stirring. Filter each time before use.

2.3. Staining Wet Cell Preparations

Kimura stain ***(6)***: Prepare stock solutions a–d which have a longer shelf life individually than the complete stain.

a. Dissolve 0.05 g toluidine blue in 22 mL 90% ethanol, add 1.8 g NaCl, and dilute to 100 mL with distilled water.
b. 0.03% light green stain SF yellowish (certified stain CI 42095) in distilled water.
c. Saturate 50% ethanol with saponin (Sigma, Poole, UK).
d. 0.07 M sodium phosphate, pH 6.4.

The complete stain can be made by mixing 11 mL (a), 0.8 mL (b), 0.5 mL (c), and 5 mL (d). This stain is stable for 3–4 wk at 4°C.

2.4. Peroxidase Method

1. Peroxidase blocking solution: 0.5% H_2O_2, 0.1% NaN_3 in methanol. Sodium azide is highly toxic, so PPE should be worn.
2. PBS-Albumin: 1% fraction V bovine serum albumin in phosphate-buffered saline.
3. Monoclonal antibody response to tryptase, AA1 ***(9)*** (Dako, Glostrup, Denmark, or AbD serotec, Oxford, or Lab Vision, Freemont, CA).
4. Monoclonal antibody to chymase, CC1 ***(10)*** (serotec or Lab Vision).
5. Negative control monoclonal antibody to *Aspergillus niger* glucose oxidase, X931 (Dako).
6. Wash buffer A: 0.4 M NaCl, 50 mM Tris, 0.05% Tween-20, pH 8.5.
7. Wash buffer B: 0.15 M NaCl, 50 mM Tris, 0.05% Tween-20, pH 8.5.
8. Wash buffer C: 50 mM Tris, 0.1% Tween-20, pH 8.5.
9. Biotinylated goat antiserum to mouse immunoglobulins and Extravidin®-peroxidase conjugate (EXTRA 2 kit, Sigma).
10. Acetate buffer: 0.1 M sodium acetate, pH 5.0.
11. AEC stock solution: 10 mg/mL 3-amino 9-ethylcarbazole in dimethylformamide. This stock can be stored in the dark for up to 1 wk at 4°C. Avoid polystyrene vessels because these are attacked by the solvent.
12. AEC substrate solution: 1 mL AEC stock, 19 mL acetate buffer, 3.3 µL 30% H_2O_2. Filter immediately before use.
13. Mayer's hemalum (BDH, Poole). Filter each time before use.

2.5. Alkaline Phosphatase Method

1. TBS-albumin: 1% fraction V bovine serum albumin in Tris-buffered saline.
2. Biotinylated goat antiserum to mouse immunoglobulins and Extravidin®-alkaline phosphatase conjugate (EXTRA 2A kit, Sigma).
3. Alkaline phosphatase buffer: 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4.
4. 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt; BCIP) in dimethylformamide. Store at 4°C, avoiding polystyrene vessels.
5. 75 mg/mL *p*-nitrotetrazolium blue (NBT) in 70% dimethylformamide, can be stored at 4°C.
6. Alkaline phosphatase substrate solution: add 75 µL BCIP stock and 100 µL NBT stock to 20 mL alkaline phosphatase buffer containing 0.25 mg/mL levamisole to block most endogenous alkaline phosphatases.

2.6. Equipment

For immunohistochemistry, it is imperative that the sections do not dry out or else substantial nonspecific staining will occur. Therefore, incubations lasting more than 5 min should take place on a slide tray which holds the slides above a pool of tap water. A close fitting lid for the tray allows a humidified atmosphere to be maintained.

3. Methods

3.1. Fixation and Processing

1. Fix tissues as soon as possible after removal to preserve architecture.
2. If the tissue sample is large, cut the tissue into smaller pieces (1 cm^3 or less) to aid penetration of the fixative. Handle the tissue with care and cut with a scalpel to minimize crush artifacts.
3. Add a generous amount of neutral buffered formalin or Carnoy's fixative and leave to fix overnight at room temperature (*see* **Note 2**). Carnoy's fluid erodes plastics, so use glass vessels with this fixative.
4. Remove the fixative and dispose of according to local regulations. Carnoy's fluid can first be neutralized by the addition of $NaHCO_3$. Add PBS and mix gently on a spiral or rotary mixer at 4°C for 1–2 h to wash out the fixative.
5. At intervals of an hour or more, dehydrate the specimens through a series of graded alcohols (70, 90, 100, and 100%) with gentle mixing at 4°C.
6. Embed the tissues in paraffin wax and cut into 4 to 6-µm sections. For immunohistochemistry, sections can be wrapped and stored at 4°C for up to 2 wk before staining.

3.2. Histochemical Staining of Mast Cells

1. Remove the slides from the refrigerator and allow to come to room temperature before unwrapping them and warming them in an incubator at 37°C for a few minutes.
2. Remove the wax from the sections by immersion in Histoclear for 5 min, then transfer to another batch for a further 5 min.
3. Rehydrate the sections through a series of alcohols in the order: 100, 100, 90, and 70% ethanol, then transfer to distilled water, spending 5 min in each.
4. Place the sections in Alcian blue or toluidine blue solution between 30 and 150 min.
5. Dehydrate the sections very quickly in 70, 90, and 100%, and a brand new batch of absolute ethanol. It is crucial that dehydration is rapid; aim for 1 s in each of the alcohols.
6. Mount a coverslip using DPX mountant. DO NOT use aqueous mounting medium or staining will be lost. Alcian blue gives a pale blue staining of mast cells whereas toluidine blue produces an intense blue/purple color.

3.3. Staining of Wet Cell Preparations

Kimura stain ***(6)*** can be used to identify mast cells in dispersed cell preparations; staining is based on toluidine blue metachromasia. The stain also contains saponin which lyses red blood cells.

1. Incubate 90 µL cell suspension with 10 µL Kimura stain for 5 min at 37°C.
2. Examine the cells using a hemocytometer. Mast cells stain a bright cerise color.
3. If erythrocytes are too numerous, they will not all lyse and may interfere with staining. Therefore, before counting, dilute the cells further or lyse the red blood cells with hypotonic saline or 0.85% ammonium chloride solution.

3.4. Immunohistochemistry: Peroxidase Method

Note: sodium azide inhibits the peroxidase enzyme used in this method, so MUST NOT BE USED as a preservative for the buffers.

1. Remove the wax and rehydrate the sections as described in **Section 3.1.** (steps 1–3).
2. Inhibit endogenous peroxidase activity with the peroxidase blocking solution for 10 min.
3. Block nonspecific protein-binding sites with PBS-albumin for 10–30 min.
4. Discard the PBS-albumin and wipe around the sections with a piece of tissue. This minimizes the spread of liquid and therefore reduces the amount of antibody required to stain the section and the chances of the section drying out.
5. Apply 100 µL primary antibody diluted in PBS-albumin to the section and incubate for 90 min at room temperature. It is advisable to titrate the antibodies before use; we find that concentrations of monoclonal antibodies of around 1 µg/mL usually give good results. Remember to include a negative control antibody in the staining run.
6. Collect the slides in a rack and wash in a trough of running tap water for 5 min. Avoid a direct jet which may dislodge the sections. Next, wash the slides in wash buffers A, B, and C (5 min each).
7. Wipe around the sections and apply 100 µL of the biotinylated secondary antibody diluted 1/20 in PBS-albumin. Incubate the slides for an hour at room temperature.
8. Wash the slides as described in step 6.
9. Wipe around the sections and apply 100 µL Extravidin®-peroxidase diluted 1/20 in PBS-albumin. Incubate the slides for an hour at room temperature.
10. Wash the slides as described in step 6. Whilst the sections are being washed, prepare the AEC substrate solution.
11. Apply the AEC substrate solution for 3–4 min or until color develops. In practice this means starting to collect slides in a rack after 2 min; staining can continue with the slides in a vertical position.
12. Wash the sections in running tap water for 5 min, then rinse briefly in distilled water.
13. Counterstain in Mayer's hemalum for 2 min, then wash as described in step 12. DO NOT USE hematoxylin which must be blued in acid alcohol as this will remove the AEC.
14. When the sections have dried completely, mount a coverslip using aqueous mounting medium. DO NOT USE xylene-based medium such as DPX as this will solubilize the AEC stain. The AEC gives a reddish-brown staining pattern.

3.5. Immunohistochemistry: Alkaline Phosphatase Method

Phosphate ions inhibit the enzyme used in this staining protocol, so a TBS-albumin rather than a PBS-albumin solution is employed.

1. Perform steps 1 and 3–8 in **Section 3.4**. Step 2 is omitted because there is no need to block endogenous peroxidase, as a peroxidase substrate will not be used.
2. Wash the slides as described in **Section 3.4.**, step 6.

3. Wipe around the sections and apply 100 µL of a 1/20 solution of Extravidin®-alkaline phosphatase in TBS-albumin for 30 min.
4. Wash the slides as in step 2 above. Whilst the slides are being washed, prepare the alkaline phosphatase substrate solution. Levamisole is an inhibitor of many endogenous alkaline phosphatases, but not the intestinal form generally used for conjugation.
5. Apply the substrate solution for 10–15 min.
6. Wash the sections in running tap water for 5 min, then rinse briefly in distilled water.
7. Counterstain with Mayer's hemalum for 2 min, then wash the slides as in step 2.
8. Because the blue/black precipitate formed is insoluble in water or xylene, either aqueous or xylene-based media can be used to mount a coverslip.

3.6. Double-Labeling Immunohistochemistry

It is difficult to cut paraffin wax sections thin enough to get several sections through a single cell. Therefore, for colocalization of antigens in wax-embedded tissue, it is necessary to adopt a double-labeling procedure. Some workers combine fluorescent and chromogenic staining. However, we prefer to use different colored stains as this allows counterstaining and sections can be examined and photographed a long time after staining. If the two primary antibodies to be used come from different species, secondary antibodies conjugated to different enzymes can be used. However, where both primary antibodies are of mouse origin, it is necessary to have different labels conjugated directly to them. In the following section, we describe the detection of tryptase and chymase using mouse monoclonal antibodies. As chymase is the less abundant antigen ***(17)***, the chymase antibody should be biotinylated because more than one biotin molecule can be conjugated to each immunoglobulin, thus providing an amplification step. The tryptase antibody can be directly labeled with horseradish peroxidase. To allow distinction between the different antibodies, contrasting chromogens should be used. In the example below, tryptase antibody binding causes reddish-brown staining whereas binding of chymase antibodies produces a blue/black color. Care must be exercised to get the correct balance of staining of the two target proteins; antibody concentrations should be titrated and incubation times selected carefully.

1. Dissolve the wax, rehydrate the tissue, and block peroxidase activity as described in **Section 3.4.**, steps 1–2.
2. Block nonspecific protein-binding sites with TBS-albumin for 10–30 min. Do not use PBS-albumin as the phosphate will interfere with the alkaline phosphatase enzyme used later.
3. Wipe around the sections and apply (a) 1/100 peroxidase-conjugated antitryptase antibody (*see* below), (b) 1 µg/mL biotinylated antichymase antibody, (c) 1 µg/mL negative control antibody, or (d) both antibody conjugates together. Perform dilutions in TBS-albumin and incubate for 3 h at room temperature.

4. Wash the sections as described in **Section 3.4.**, step 6.
5. Apply the filtered AEC substrate solution for 5 min.
6. Wash the sections as described in **Section 3.4.**, step 6.
7. Wipe around the sections and apply 1/20 Extravidin®-alkaline phosphatase in TBS-albumin for 1 h.
8. Wash the sections as described in **Section 3.4.**, step 6.
9. Apply the BCIP/NBT substrate solution for 10 min.
10. Wash the slides in running tap water for 5 min, rinse in distilled water, then counterstain in Mayer's hemalum for 2 min, and wash with tap water and distilled water again.
11. Because the AEC is soluble in alcohol, aqueous medium should be used to mount coverslips.

3.7. Cytocentrifuge Preparations

It is possible to store cell suspensions for staining at a later date by making cytocentrifuge preparations (Cytospins).

Establish the number of cells using a hemocytometer, then adjust to 1,000,000 per mL using a physiological strength buffer.

Assemble the cytocentrifuge according to the manufacturer's instructions, then load 100 µL of cell solution per slide, and centrifuge for 5 min at 500 rpm (28*g*).

Remove the slides, taking care not to smear the cells. Allow the cells to air dry, then fix in methanol or acetone for 1 min.

For histochemical staining, the cytospins can be stored at room temperature. For immunocytochemistry, wrap them tightly in aluminum foil and store at 4°C.

4. Notes

1. Alcian blue is very difficult to dissolve, so prepare it well in advance. Add the stain to the acid very slowly with continual magnetic stirring. Do not add any more stain until the last batch has dissolved completely, otherwise the dye will come out of solution permanently. Be prepared for this to take all day. Filter each time before use.
2. If antibodies against tryptase and chymase other than those described in this chapter are to be employed, then the antigen may be sensitive to fixation. Therefore it may be advisable to fix tissue samples in 85% ethanol for 1 wk at 4°C instead of formalin, then dehydrate them through 90% and absolute ethanols. However, care must be taken in handling these specimens before embedding, as 85% ethanol may be less efficacious than many other fixatives in eliminating biohazards. Fixation in ethanol alone will lead to some tissue shrinkage.

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