Histochemistry of Bone Marrow Aspirations

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ABSTRACT

The accuracy of diagnosis of hematological diseases from bone marrow aspirates and peripheral blood smears is considerably improved by use of histochemical stains. As hematopoietic cells differentiate and mature from the pluripotent stem cell and are committed to a particular cell line, the committed cell lines produce different substances, particularly enzymes, which can be identified by various histochemical stains.

Histochemical stains are not diagnostic, but are aids in diagnosis, because various cell lines may produce similar substances and because the stains lack absolute specificity. A discussion of the use of histochemical stains for the diagnosis of leukemias is presented. Complete procedures for Prussian blue stain for iron, peroxidase, Sudan black B, naphthol AS-D chloroacetate esterase, nonspecific esterase, periodic acid Schiff (PAS), acid phosphatase, toluidine blue and alkaline phosphatase stains are given in the appendix.

Introduction

The usual stain for bone marrow aspirate and peripheral blood smears is Wright's or the Wright-Giemsa stain. With these stains, nucleated cell identification is determined from cellular morphology and polychromatric staining characteristics. However, as maturation of cell lines from the original stem cell becomes altered and/or delayed, as observed in leukemias, peripheralizing lymphomas and multiple myelomas, positive identification of the nucleated cell lines from polychromatic stained morphological characteristics becomes increasingly difficult. As cell lines become committed from the stem cell precursors and mature, differences between the cell lines in cell constituents, particularly enzymes, occur. When these differences become adequate to be detected by histochemical staining methods, a more nearly accurate identification of the cell line can be made. Obviously, if the cell lines are so near the original stem cell that adequate differences in cell constituents do not exist, then histochemical stains are not helpful in differentiating the various cell lines. Moreover, since the various cell lines may have overlapping histochemical staining characteristics, histochemical stains are an aid to diagnosis and not diagnostic within themselves.
Substances in Hematopoietic Cells That Can Be Identified by Histochemical Stains

IRON

Iron is stored in the bone marrow principally in the form of ferritin and hemosiderin. Ferritin is not visible microscopically, but hemosiderin appears as a golden pigment often in the form of granules. The ferric iron of hemosiderin is stained as a blue pigment by the Prussian blue reaction. Prussian blue stainable iron is present in reticulum cells, macrophages, hemohistiocytes, siderocytes, the mitochondria of ringed sideroblasts and rarely in plasma cells or leukocytes.

PEROXIDASE

Peroxidase is present in large amounts in the azurophilic (primary) granules of myeloid cells and eosinophils. Peroxidase is present at much lower concentrations in monocytes. Basophils and lymphocytes do not contain peroxidase.

PHOSPHOLIPIDS

Phospholipids are present in most cells, but are found in highest concentration in the membranes of the azurophilic and specific granules of myeloid cells and in lower concentration in the granules of monocytes.

ESTERASES

Many esterases are present in most hematopoietic cells. However, by selection of substrates and reaction conditions, the esterase reaction is useful in identification and differentiation of myeloid and monocytic cell lines.

GLYCOGEN

Glycogen is present in large quantities normally in myeloid cells with increased quantities appearing in the more mature cells. Basophils, eosinophils, megakaryocytes and platelets also contain large amounts of glycogen. Smaller quantities of glycogen are present in monocytes. Glycogen is normally not detectable by histochemical means in other normal hematopoietic cells, but it is often observed in abnormal erythroid, lymphoid, and plasmacytoid cells.

HEPARIN

Heparin is present in basophils and mast cells. Its identification is occasionally useful in positively differentiating between immature basophils, promyelocytes and individuals with Alder-Reilly anomaly.

ACID PHOSPHATASE

Acid phosphatase is present in all cells of the granulocytic series with the exception of mature basophils. Most acid phosphatases are inhibited by L(+)-tartaric acid. Tartrate resistant acid phosphatase is usually present in the malignant cells of hairy cell leukemia and in the malignant lymphoid cells of some T-cell lymphomas and lymphocytic leukemias.

ALKALINE PHOSPHATASE

In the myeloid series, alkaline phosphatase is first noted in myelocytes and is associated with the development of specific (secondary) granules. In mature human neutrophils, the enzyme is associated with a microsomal fraction and not with either type of granules. The enzyme is not found in other hematopoietic cells.

Histochemical Stains As a Diagnostic Aid

PRUSSIAN BLUE IRON STAIN

The Prussian blue iron stain stains the ferric iron in hemosiderin which is ob-
served microscopically as a blue pigment.\textsuperscript{3,5,41} In bone marrow aspirates, the amount of iron present can be assessed in a crushed spicule, but determination that the iron is intracellular is essential. This is most easily determined by observation of macrophages in a smear of bone marrow aspirate as indicated in figure 1A. Overall iron assessment is most easily made by observation of a stained bone marrow clot section or biopsy. Confirmation of ringed sideroblasts as an aid to the diagnosis of sideroblastic anemia is most easily made on a bone marrow aspirate smear as indicated in figure 1B. Siderotic granules in the peripheral blood stain positively. Hence, the Prussian blue iron stain allows an assessment of iron stores and dyserthropoiesis.

**Peroxidase Stain (PEROX) for Myeloid Cells**

A positive peroxidase stain highly suggests the presence of a myeloid cell line\textsuperscript{18,30,35} and definitively separates the myeloid from the lymphoid cell line. With positive peroxidase staining, the only possible confusion is with the monocytoid cell lines. Monocytes rarely stain positively with the peroxidase stain used in this laboratory and then only extremely weakly. Since the peroxidase is present in the azurophilic granules, a strongly positive reaction is observed in promyelocytes and often in blasts in which granules are not observed with a Wright's stain as indicated in figure 1C. Auer rods usually stain positively as shown in figure 1D.

**Sudan Black B Stain (SSB) for Myeloid Cells**

Sudan Black B stains phospholipids in the azurophilic and specific granules of myeloid cells (figure 1E) but rarely stains the granules in monocytes. It does not stain lymphocytes. Auer rods usually stain positively.

**Esterase Stains for Myeloid and Monocytoid Cells**

The choice of substrate and reaction conditions for esterase reactions makes esterases an invaluable aid in discriminating between myelocytic, monocytic and lymphocytic cell lines.\textsuperscript{26,33,43} Apparently the production of esterases is associated with early granule development, for positive reactions can be observed before granules are observed with Wright's stain.

**Naphthol AS-D Chloroacetate Esterase (NASD) for Myeloid Cells.** Positive naphthol AS-D chloroacetate esterase staining of leukocytes is extremely suggestive of the myeloid cell line. The NASD esterase stain is rarely, if ever, positive in monocytes. Staining of myeloid cells is indicated in figure 1F. Mast cells are positive,\textsuperscript{17} but lymphocytes, megakaryocytes, plasma cells, and normoblasts appear to be consistently negative. Auer rods often stain positively.

**Nonspecific (α-Naphthyl Acetate) Esterase (NSE) for Monocytes.** The most positive nonspecific esterase staining is observed in monocytes as indicated in figure 1G. However, megakaryocytes, platelets and macrophages also stain positively, but the staining is weaker.\textsuperscript{33,43} Weak positivity may also be observed in normoblasts and myeloid cells. Slight focal positivity is also often observed in lymphocytes, but may be more artifact than true positivity. This esterase is readily inhibited by fluoride and many insecticides.

**Periodic Acid-Schiff Stain (PAS) for Glycogen**

Periodic acid oxidizes vicinal hydroxyl groups (or potential hydroxyl groups such as the amino groups) to aldehydes, which will then react with a Schiff's base. Although the reaction is not specific for glycogen, glycogen is most often the compound yielding PAS positivity in tissue.
cells. Mucopolysaccharides and glycoproteins may also result in a positive PAS stain. Identity of glycogen can be made by treating the tissue with salivary diastase and noting the absence of PAS positivity, but this is not 100 percent specific.

The red PAS positivity as observed in hematopoietic cells can be described in four ways. First, the cytoplasm of the cell may be clear and stain uniformly looking much like pink or red glass. This staining characteristic is most often observed in erythrocytes of erythroleukemia and sometimes in iron deficiency or sideroblastic anemias as indicated in figure 1H. Normal lymphocytes rarely stain this way.

The second type of staining pattern is a finely diffuse PAS positivity owing to very fine pinpoints or streams of positivity which are beyond the discriminatory power of the light microscope. The entire cytoplasm or only bits of it may be PAS positive. This finely diffuse PAS positivity is most often observed in myeloblasts and monocytes, but may be observed in plasma cells, macrophages, and other cells containing mucopolysaccharides or glycoproteins.

The third type of staining pattern observed is the coarsely diffuse PAS positivity. In this staining pattern, the PAS positivity is approximately the size of the azurophilic granules seen in myeloid cells. This PAS positivity has diffuse edges and definite clear cytoplasm appears between the PAS positive spots. This type of PAS positivity is most often observed in myeloid cells beyond the blast stage (the granules do not stain), platelets and megakaryocytes. Rarely, a normal lymphocyte may exhibit coarsely diffuse positivity peripherally. Five to 95 percent of the lymphocytes in approximately 70 percent of the cases of chronic lymphocytic leukemia, lymphosarcoma cell leukemia and lymphomas may exhibit this type of positivity. Rarely, lymphoblasts in acute lymphocytic leukemia and lymphocytes in infectious mononucleosis exhibit coarsely diffuse PAS positivity.

The fourth type of staining pattern observed is blocky PAS positivity. In this pattern, the PAS positivity is opaque and encompasses large areas up to half of the cytoplasm. In approximately 80 percent of the cases of acute lymphocytic leukemias, five to 95 percent of the blasts exhibit blocky PAS positivity as shown in figure II. Blocky PAS positivity may also be observed in lymphomas as shown in figure 1H, particularly in the T cell and Sezary cell types.

TOLUIDINE BLUE STAIN FOR HEPARIN

Toluidine blue stains heparin and is useful in the identification of basophils and mast cells. Rarely, it is useful to differentiate between promyelocytes and the immature basophils that are observed in basophilic leukemia and chronic myelogenous leukemia in blast crisis. Toluidine blue stain may also be useful to differentiate between basophils and neutrophils with extreme toxic granulations or Alder-Reilly anomaly.

ACID PHOSPHATASE STAIN (ACP)

Since acid phosphatases are present in most hematopoietic cells, an acid phosphatase stain alone is of little value. Acid phosphatase can be separated electrophoretically into isoenzymes. Six of these acid phosphatase isoenzymes are inhibited by L (+) tartric acid. However, acid phosphatase isoenzyme #5, which is normally found in hairy cell leukemia and a few T-cell malignancies, is not inhibited by L (+) tartaric acid. This is of great clinical importance since the treatment and prognosis of hairy cell leukemia are vastly different from all other lymphoid malignancies and the diagnosis must be made with certainty. In most cases of hairy cell leukemia, treatment with cytotoxic drugs is contraindicated. Also the prog-
FIGURE 1. Histochemical stains of hematopoietic cells.

A. Prussian blue iron stain. Erythroblastotic island containing iron laden hemohistiocyte nurturing surrounding erythroblasts in normal bone marrow aspirate.

B. Prussian blue iron stain. Ringed sideroblast with blue stained iron containing mitochondria in bone marrow aspirate in 54 year old male with early myelofibrosis.

C. Peroxidase stain. Myeloid cells at various stages of maturity with positive golden brown peroxidase positivity in peripheral blood of 33 year old female with chronic myelocytic leukemia. Note obvious blast containing two nucleoli and slight peroxidase staining on one side.

D. Peroxidase stain. Myeloblasts showing variable peroxidase positivity and auer rods in peripheral blood of 45 year old male with acute myelomonocytic leukemia.

E. Sudan black B. Myeloid cells at various stages of maturity exhibiting positive black SBB positivity in same case as C.

F. Naphthol AS-D chloroacetate esterase. Myeloblasts exhibiting fine pink NASD positivity in cytoplasm of bone marrow aspirate from 28 year old male with acute myelocytic leukemia.

G. Non-specific esterase stains. Monoblasts exhibiting positive brown NSE staining in peripheral blood of 26 year old male with acute monocytic leukemia.

H. Periodic acid Schiff: Note clear glass appearance of PAS positivity in erythrocytes in a case of erythroleukemia in a 58 year old male.

I. Periodic acid Schiff. Lymphoblasts exhibiting extensive blocky PAS positivity in bone marrow aspirate of 27 year old male with peripheralizing lymphoma. Note positively staining platelets.

J. Periodic acid Schiff. Lymphoblasts exhibiting blocky PAS positivity in peripheral blood of 34 year old male with acute lymphocytic leukemia.

K. Acid phosphatase without tartrate. Lymphoblasts exhibiting positive maroon staining in bone marrow aspirate of same case as J. With tartrate inhibition, this stain was totally negative.

L. Acid phosphatase with tartrate inhibition. Extensive positive ACP staining with tartrate inhibition in frozen section of spleen from 26 year old male with hairy cell leukemia. Note normal lymphoid follicle with almost no positivity. The positive hairy cells were observed in the blood vessels, but did not penetrate the capsule.
nosis in this unusual malignancy may be 20 years or more. The acid phosphatases are relatively unstable and, hence, an acid phosphatase stain without tartrate must always be done to assure that the acid phosphatase activity is present which can be inhibited by tartrate. Acid phosphatase stains with and without tartrate inhibition are indicated in figures 1K and 1L.

**Alkaline Phosphatase Stain (LAP)**

The alkaline phosphatase stain has its greatest utility as an aid in differentiating between a leukemoid reaction and chronic myelogenous leukemia. This is especially important if a leukemoid reaction and chronic myelogenous leukemia are present simultaneously. Only mature neutrophils and bands should be scored by the Kaplow scoring method. The normal range is extremely great. Neutrophils with toxic granulations, as seen in the peripheral blood of patients with pyogenic infections, tend to have an increased leukocyte alkaline phosphatase activity. In chronic myelogenous leukemia, the alkaline phosphatase score of neutrophils is usually low, but a concurrent pyogenic infection with only a few neutrophils having a high score will result in a Kaplow score within the normal range. The alkaline phosphatase stained smears must be assessed for the possibility of such biclonal distribution of neutrophils. The clinical findings in such cases are of immense importance.

**Diagnosis of Leukemias**

With Wright’s stained preparations of blood and marrow, approximately 90 percent of all human leukemias can be identified with great accuracy as to their histologic types. An additional five percent can be identified with reasonable accuracy, but the remaining five percent cannot be classified accurately. With histochemical stains, the accuracy of histologic diagnosis can easily be confirmed beyond any reasonable doubt in the first 90 percent, and with great accuracy in an additional eight to nine percent. Approximately one to two percent of all leukemias are comprised of cells so primitive that no differential histochemical markers are present. Therefore, they can only be classified as undifferentiated or stem cell leukemias.

Peroxidase, Sudan black B and naphthol AS-D chloroacetate esterase reactions tend to parallel each other and to be positive concurrently and to serve to identify myeloid cells. Auer rods usually give a positive staining reaction with all three stains, but exceptions are found. In addition, PAS positive Auer rods may be observed. A case of myeloid leukemia has been followed in which the cytoplasmic granules stained only with Sudan black B and naphthol AS-D chloroacetate esterase. In another case of granulocytic leukemia, only naphthol AS-D chloroacetate esterase activity of cytoplasmic granules could be demonstrated. Even the mature neutrophils in this latter case did not exhibit peroxidase or Sudan black B positive staining. This experience suggests that the naphthol AS-D chloroacetate esterase stain gives slightly fewer false negative results. Lymphocytic leukemias can be ruled out if any of these three stains are positive. However, all these three stains may stain cells of the monocytic series, but usually the staining reaction is only weak. Experience suggests that naphthol AS-D chloroacetate esterase is the most discriminatory staining reaction for differentiating between myeloid and monocytic leukemias. The peroxidase reaction is the least discriminatory. A strongly positive nonspecific esterase stain even with weakly positive staining by the other three stains (peroxidase, Sudan black B, ASP esterase) is overwhelming evidence for the presence of cells of the monocytic series.
Histochemical stains are not as helpful in the identification of lymphocytic leukemias for no specific substance is known that can be stained to identify positively the lymphoid cell. Blocky PAS positivity in blasts is strong evidence, but not conclusive proof that the leukemia is lymphocytic. Acute lymphocytic leukemia is diagnosed by the absence of all myeloid or monocytoid staining properties and appropriate cellular morphology. In this setting, the presence of blocky PAS positivity is considered confirmatory. Additional evidence for a lymphocytic leukemia may be obtained from B and T typing and terminal deoxynucleotidyl transferase activity. A summary of these various stains for differential diagnosis of leukemias is given in table I.

Summary

Histochemical procedures are presented in the appendix. The procedures are given exactly as used in this laboratory and in our hands give consistently good results. Slight modifications from the referenced procedures have been made. All of these procedures can be performed in any pathology laboratory with minimal or no extra equipment. However, depending upon the hematological case workload, one may wish to institute only certain selected procedures. The iron stain, the PAS stain and a stain for myeloid cells either peroxidase or sudan black B (overall the more consistent), would give adequate resources for over 95 percent of the diagnostic hematological workups with minimal outdated of reagents. The next stain to add to one’s histochemical profile would be the nonspecific esterase stain for monocyte identification. The acid phosphatase is most useful in the diagnosis of hairy cell leukemia in patients with lymphoid disease since such a diagnosis will not only dictate a marked change in the usual treatment of malignant lymphomas, but consequently will greatly improve the prognosis. The measurement of leukocyte alkaline phosphatase activity by histochemical

TABLE I
Summary of Histochemical Stains for Differential Diagnosis of Leukemias

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<thead>
<tr>
<th>Leukemia</th>
<th>PEROX</th>
<th>SBB</th>
<th>NASD</th>
<th>NSE</th>
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+ = Indicates degree of positive reaction.
- = Indicates no staining.
± = Indicates variable degrees of staining.
* = Blocky periodic acid Schiff positivity often observed.
§ = Blocky or coarsely diffuse positivity often observed.
† = Positive staining is reported in T-cell leukemia and lymphoma.
PEROX = Peroxidase stain.
SBB = Sudan black B.
NASD = Naphthol AS-D chloroacetate esterase.

ACP = Acid phosphatase stain.
AMML = Acute myelomonocytic leukemia.
ARM = Acute myelocytic leukemia.
ALL = Acute lymphocytic leukemia.
AUL = Acute undifferentiated leukemia.
HCL = Hairy cell leukemia.
CML = Chronic myelocytic leukemia.
CLL = Chronic lymphocytic leukemia.

ACP = Acid phosphatase stain.
AMML = Acute myelomonocytic leukemia.
ARM = Acute myelocytic leukemia.
ALL = Acute lymphocytic leukemia.
AUL = Acute undifferentiated leukemia.
HCL = Hairy cell leukemia.
CML = Chronic myelocytic leukemia.
CLL = Chronic lymphocytic leukemia.

ACP = Acid phosphatase stain.
AMML = Acute myelomonocytic leukemia.
ARM = Acute myelocytic leukemia.
ALL = Acute lymphocytic leukemia.
AUL = Acute undifferentiated leukemia.
HCL = Hairy cell leukemia.
CML = Chronic myelocytic leukemia.
CLL = Chronic lymphocytic leukemia.
methods is also a most useful stain. Toluidine blue stain is so cheap, long-lasting and simple to perform, it could be added to any clinical laboratory for the few rare cases in which it is needed. Finally, the naphthol AS-D chloroacetate esterase stain is a truly beautiful stain with good discriminatory powers, but it is more difficult to perform and seldom adds more information to that already observed with the peroxidase or sudan black B stain.

A discussion of the use of histochemical stains in the diagnoses of leukemias has been presented.

Appendix:

Procedures for Histochemical Stains

Freshly distilled or deionized water should be used throughout unless otherwise specified. The only equipment needed is generally found in most pathology laboratories and includes a refrigerator with freezer, pH meter, water bath, milligram balance, magnetic stirrer, pipettes, graduated cylinders, screw cap Coplin jars, volumetric flasks, forceps and a water tap. All glassware must be thoroughly cleaned, rinsed with distilled water and dry before use. Most reagents and stains can be prepared and stored in Coplin jars. The highest purity chemicals available should be used. All reactions are run at room temperature unless otherwise specified. Although storage conditions and times of expected useful life of reagents are given, discard any reagent which contains any suspected bacterial growth or other signs of deterioration. Precautions for handling acids and flammable materials are not given since any competent technologist should be aware of these precautions. Since Wright's or Wright-Giemsa stains are used almost universally in hematology laboratories, instructions for their use are not given.

Reagents Required for Stains

**Store at Room Temperature**


**Store in Refrigerator**

1. Acetate solution, 2.5M (386-3);* 2. Citrate concentrate, 0.385M (365-1);* 3. Fast garnet GBC salt (386-15);* 4. Hydrogen peroxide, 3 percent; 5. Naphthol AS-D chloroacetate esterase solution (386-4);* 6. Schiffs reagent (6973);† and 7. L (+) Tartrate solution, 0.67M (386-2).*

**Store in Freezer**


Specimens and Control Slide for Staining Fresh Specimens of Peripheral Blood, Bone Marrow Aspirate

Fresh specimens of bone marrow aspirate, crushed spicules, peripheral blood or other body fluids may be used for most stains. The specimen may be collected without anticoagulant or collected in heparin or ethylenediamine tetraacetic acid (EDTA). No anticoagulant should be used in collecting specimens for leukocyte alkaline phosphate (LAP) staining. Heparin should not be used in specimen collection for toluidine blue staining.

All specimens must be fresh and prepared as soon as possible on slides, then air dried and stored in the dark in a cool, dry, dust-free environment except as follows. Staining should be performed as soon as possible.

Little or no loss of staining capacity for iron, PAS, SBB or toluidine blue is noted after years of storage. PEROX and NASD activity persists for at least three weeks and often for years. NSE staining should be performed within a week, but some activity persists for several months. LAP or ACP staining should be performed immediately if possible or stored in a refrigerator up to one week. Positive activity may be observed in specimens that are placed in a freezer immediately after preparation for up to one year.

Control slides must be stained for each different staining technique. Control slides must be run with each batch of specimens and must stain positively. Iron control slides are best prepared from crushed bone spicules which contain a quantity of iron. Fresh peripheral blood smears containing an adequate number of neutrophils are best used as controls for the PAS, PEROX, SBB and NASD stains. Fresh peripheral blood smears containing an increased number of monocytes are best used as controls for the NSE stains. Fresh peripheral blood smears should be used as controls for the ACP stains both

* Sigma Chemical Company Catalog number.
† Fisher Scientific Company Catalog number.
with and without tartrate. In addition, if possible, frozen peripheral blood smears or frozen sections of lymph nodes or spleen from a patient with hairy cell leukemia should be used as controls for the ACP stain both with and without tartrate. Such slides from a case of hairy cell leukemia have been stored in this laboratory in a -40°F freezer for up to two years without significant loss of ACP activity. LAP control smears are made from peripheral blood obtained by finger stick. The best blood smears are made from laboratory in a -40° freezer for up to two years with —

Prussian Blue Iron Stain

**PRINCIPLE**

The ferrocyanide ion reacts with ferric ions to produce a brilliant blue pigment called Prussian blue which has the approximate composition of KFe(Fe(CN)_6).H_2O. This pigment is identical to Turnbull's blue which is made by reacting ferricyanide ions with ferrous ions.

**REAGENT PREPARATION**

_Ferrocyanide—HCl Reagent_. A one percent potassium ferrocyanide in 0.1N HCl is prepared by dissolving 0.5 g of potassium ferrocyanide in 47.5 ml of water and adding 2.5 ml of 2N HCl. Mix well. (Stable for three months at 25°).

_Safranin (0.1 Percent) (or Eosin)_ Dissolve 0.100 g of safranin (or eosin) in 100 ml of water. (Stable for one week at 25°).

**PROCEDURE**

Fix air dried specimen and control slides for two minutes in methanol (100 percent). Remove slides from methanol and blot off excess methanol. Place slides in ferrocyanide—HCl reagent for one hour. Remove slides and rinse in tap water for one minute. Counterstain with 0.1 percent safranin (or eosin) for 10 seconds. Rinse slides in tap water for one minute. Dry in air and coverslip if desired.

**RESULTS AND COMMENTS**

The presence of iron is indicated by a bright blue pigment. Assessment of iron stores in bone marrow is made by estimating the quantity of blue stain present in a spicule as compared to the normal control. That the iron is intracellular is best observed in isolated macrophages or in erythroblastotic islands. Ringed sideroblasts contain five or more positively stained mitochondria around the nucleus. Siderocytes may have single or multiple blue granules.

Wright's stains may be counterstained by the Prussian blue iron stain. The iron stain may be counterstained by the FAS stain.

Peroxidase Stain

**PRINCIPLE**

Peroxidase oxidizes o-tolidine base by transferring hydrogen with its electron from the amino group to hydrogen peroxide to yield water and oxidized o-tolidine. If the reaction occurs at an acid pH, the oxidation is partial and a blue dimer precipitates at the site of reaction. At an alkaline pH, the o-tolidine is completely oxidized yielding an insoluble golden brown monomer which precipitates at the site of reaction.

**REAGENT PREPARATION**

_Fixative, 4 Percent Formaldehyde in Ethanol_. Add five ml of 40 percent formaldehyde to 45 ml of absolute ethanol. (Stable for one week at 25°).

_Peroxidase Reagent_. To 30 ml of absolute ethanol in a Coplin jar add 20 ml of distilled water and mix. Add 250 mg o-tolidine and mix for 5 minutes. (A slight precipitate may remain in the solution without effect.) Add 0.1 ml of 3 percent hydrogen peroxide. Use fresh and for only one batch of specimens.

**PROCEDURE**

Fix specimen and control slides for 30 seconds in 4 percent formaldehyde in ethanol. Place slides in the Peroxidase reagent for five minutes. (Discard reagent after use.) Rinse slides thoroughly with tap water for one minute. Allow slides to air dry. Counterstain with Wright's or Wright-Giemsa stain by the usual method and coverslip if desired.

**RESULTS AND COMMENTS**

Peroxidase activity is observed as a golden brown pigment in the granules which, depending upon their peroxidase activity, may be extremely bright as observed in promyelocytes and eosinophils, a few scattered granules as observed in some myeloblasts or very faint as sometimes observed in monocytes.

Other counterstains may be used, but Wright's has the advantage that except for the positive peroxidase activity, the staining is almost identical to that of Wright's stain alone.

Ortho-tolidine is used in place of benzidine base or benzidine hydrochloride since benzidine was listed by the Federal Register in 1974 as a carcinogen and concentrations greater than 0.1 percent cannot be used without rigid safety precautions. If desired, benzidine base, 150 mg, can be used in place of o-tolidine. The structural formula of o-tolidine, 4,4'-diamo (3, 3'dimethyl) biphenyl, is identical to benzidine, 4,4'diaminobiphenyl, with the exception that o-tolidine contains two methyl groups. Another alternative to the use of benzidine has been reported by Kaplow in which 3-amino-9-ethylcarbazole is used.
Sudan Black B Stain

**PRINCIPLE**

Sudan Black B stains phospholipids principally, but also stains lightly neutral lipids as well as other substances. The stain apparently attaches to the phospholipids by adsorption since it can easily be removed by acetone.

**REAGENT PREPARATION**

**Sudan Black B (0.3 Percent) in Ethanol.** Add 0.3 g of Sudan Black B to 100 ml absolute ethanol. Allow to stand at room temperature for one to three days and shake frequently until Sudan Black B completely dissolves. Alternatively, the Sudan Black B may be ground in ethanol with a mortar and pestle and the suspension heated. (Stable for six months at 25°).

**Phosphate-Phenol Buffer.** Dissolve 16 g of crystalline phenol in 30 ml of absolute ethanol. Dissolve 0.3 g of Na₂H P0₄.12H₂O (or 0.12 g of anhydrous salt) in 100 ml of water. Mix the total volumes of the two solutions together. (Stable six months at 25°).

**Working Sudan Black B Stain.** Add 40 ml of Phosphate-phenol buffer to 60 ml of 0.3 percent Sudan Black B in ethanol. Mix well and filter. This solution may be reused repeatedly, but should be replaced when lighter staining of control slides is noted. (Stable three months at 25°).

**Ethanol, 70 Percent.** Dilute 70 ml of absolute ethanol to 100 ml with water.

**PROCEDURE**

Fix air dried specimens and control in formalin vapor for 10 minutes. While the specimens are fixing, prepare the incubation mixture as follows:

- Add 0.1 ml of 4 percent nitrite working reagent with 0.1 ml of 4 percent ammonium hydroxide in 500 ml of water. (Stable for one month at 4°C).

- Prepare the incubation mixture as follows:
  - Add 60 ml of Working Buffer, pH 6.8, to 40.8 ml of 0.1 N HCl. Adjust pH to 6.8 (using a pH meter) with 2 N HCl. If slight precipitate forms, filter. Prepare fresh for each run.
  - Naphthol AS-D Chloroacetate Solution. Dissolve 20 mg of naphthol AS-D chloroacetate in 2 ml of 0.1 N sodium barbital in water and dilute to 1000 ml. (Stable for three months at 25°).
  - Ammonia Water. Mix two to three drops of concentrated ammonium hydroxide in 500 ml of water.

**RESULTS AND COMMENTS**

Positive Sudan Black B staining is observed as discrete dark blue-black granules in the cytoplasm which often overlap the nucleus.

**Naphthol AS-D Chloroacetate Esterase Stain**

**PRINCIPLE**

The naphthol AS-D chloroacetate ester is hydrolyzed by its specific esterase to yield a naphthol complex which couples with a diazonium salt to yield an insoluble red precipitate at the site of reaction.

**REAGENT PREPARATION**

**Pararosaniline Working Reagent, 4 Percent.** A 4 percent solution is prepared by dissolving 1 g of pararosaniline in 25 ml of 2 N HCl with mild heating if necessary. Filter. (Stable for three months at 4°C). Add 0.1 N HCl. Dilute 8.4 ml of concentrated HCl or 50 ml of 2 N HCl with water to 1000 ml. (Stable for three months at 25°C).

**Working Buffer, pH 6.8.** Stock barbital solution, 40.8 ml, is mixed with 39.2 ml of 0.1 N HCl. Adjust pH to 6.8 (using a pH meter) with 2 N HCl. If slight precipitate forms, filter. Prepare fresh for each run.

**Naphthol AS-D Chloroacetate Solution.** Dissolve 20 mg of naphthol AS-D chloroacetate in 2 ml of NN-dimethylformamide in a glass test tube. Prepare fresh for each run.

**Ammonia Water.** Mix two to three drops of concentrated ammonium hydroxide in 500 ml of water.

**PROCEDURE**

Fix air dried specimens and controls in formalin vapor for 10 minutes. While the specimens are fixing, prepare the incubation mixture as follows:

- Add 0.1 ml of 4 percent pararosaniline working reagent with 0.1 ml of 4 percent nitrite working reagent on the bottom edge of a 250 ml beaker. (If the mixture turns pink, wash glassware with acid and start again). Let stand for one minute. This light yellow solution is diazotized hexazonium pararosaniline.

- **Ethanol, 70 Percent.** Dilute 70 ml of absolute ethanol to 100 ml with water.

- Add 60 ml of Working Buffer, pH 6.8, to hexazonium pararosaniline in beaker in Section A (previous paragraph) and mix well.

- Add the 2 ml of naphthol AS-D chloroacetate solution to solution from Section B (previous paragraph) and mix well.

- Counterstain with Mayer’s hematoxylin for two minutes. Rinse slides thoroughly with tap water.

- Place wet slides in a clean Coplin jar and directly filter cloudy pink buffered solution from step C into Coplin jar with slides. Incubate slides at room temperature for 45 minutes. Rinse slides thoroughly with tap water for one minute. Counterstain slides in Mayer’s hematoxylin for two minutes. Rinse slides with tap water for one minute. Rinse slides in ammonia water for 30 seconds. Rinse slides with tap water for one minute. Dry in air and coverslip if desired.

**RESULTS AND COMMENTS**

Naphthol AS-D chloroacetate esterase activity is observed as a bright red precipitate in the granules of myeloid
cells and sometimes as a very faint red precipitate in monocytoid cells. This esterase is not inhibited by fluoride.

**Nonspecific Esterase Stains**

**PRINCIPLE**

This nonspecific esterase hydrolyzes the ester, α-naphthyl acetate to yield α-naphthyl which couples with hexazonium pararosaniline to give an insoluble brownish red precipitate at the site of enzyme activity.

**REAGENT PREPARATION**

- *Sorensen’s Phosphate Buffer Solution A.* Dissolve 27.23 g of KH₂PO₄ in water and dilute to 1000 ml. (Stable for three months at 25°C).
- *Sorensen’s Phosphate Buffer Solution B.* Dissolve 28.40 g of Na₂HPO₄ (or 71.64 g of Na₂HPO₄·12H₂O) in water and dilute to 1000 ml. (Stable for three months at 25°C).
- *Sorensen’s Working Phosphate Buffer, 0.2M, pH 7.0.* Mix 17.5 ml of Solution A with 32.5 ml of Solution B. Check with pH meter and adjust to pH 7.0 if necessary. Prepare fresh before use.
- *Sodium Nitrite Working Reagent, 4 Percent.* (As prepared for NASD).
- *Pararosaniline Working Reagent, 4 Percent.* (As prepared for NASD).
- *α-Naphthyl Acetate Solution.* Dissolve 20 mg of α-naphthyl acetate in 0.6 ml of acetone in a glass test tube. Prepare fresh for each run.

**PROCEDURE**

(A) Fix air dried smears of the specimens and the controls in formalin vapor for five minutes. Wash in repeatedly replaced tap water for five minutes, then distilled water for one minute and dry in air.

(B) Prepare hexazonium pararosaniline as in step A for NASD procedure.

(C) Add 10 ml of Solution A to 40 ml of Sorensen’s working phosphate buffer, pH 7.0, to the hexazonium pararosaniline and mix well.

(D) Add the 0.6 ml of α-naphthylacetate solution to the remaining 40 ml of Sorensen’s working phosphate buffer, pH 7.0, and mix until all cloudiness disappears.

(E) Mix the solutions from steps C (hexazonium pararosaniline) and D (α-naphthyl acetate in buffer) together. Filter if cloudy and place in a Coplin jar. This is the incubation solution. Incubate the slides for 60 minutes in the incubation solution.

(F) Remove slides and rinse thoroughly in tap water for three minutes. Counterstain for ten minutes in Mayer’s hematoxylin. Remove and place in tap water for 15 minutes, then rinse for five to ten seconds under running tap water. Dry in air and coverslip if desired.

**RESULTS AND COMMENTS**

Nonspecific esterase activity is observed as a brown pigment with a reddish tinge in the cytoplasm of monocytoid cells. This esterase in monocytoid cells is inhibited by fluoride and many insecticides.

**Periodic Acid Schiff Stain (PAS)**

**PRINCIPLE**

Periodic acid oxidizes vicinal hydroxyl groups (or potential hydroxy groups such as the amino group) to aldehydes which will then react with a Schiff’s base. Although any compound with the appropriate vicinal hydroxyl groups may stain PAS positive, the PAS stain is principally used to detect glycogen. The identity of glycogen is ascertained by prior treatment of the specimen with salivary diastase which hydrolyzes glycogen and results in the absence of PAS positivity. Mucopolysaccharides, glycoproteins and other PAS positive compounds retain their positivity following such treatment with salivary diastase.

**REAGENT PREPARATION**

- *Fixative, 4 Percent Formaldehyde in Ethanol.* (As prepared for PEROX).
- *Periodic Acid, 1 Percent.* Dissolve 0.5 g of periodic acid in 50 ml of water. (Stable for one week in brown bottle at 4°C).
- *Schiff’s Reagent.* Although this reagent can be prepared in the laboratory, it is far cheaper to purchase it ready made. A screw cap Coplin jar filled with Schiff’s reagent can be repeatedly used for up to one month providing it is immediately returned to the refrigerator after use and tested for activity before each use. The test to ascertain the activity of Schiff’s reagent is to place one drop of Schiff’s reagent on a microscope slide. Add one drop of 4 percent formaldehyde. An immediate dark purplish red color indicates that the Schiff’s reagent is still good.

**PROCEDURE**

Fix specimen and control slides for ten minutes in 4 percent formaldehyde in ethanol. Rinse slides for eight minutes in running tap water. Place slides in 1 percent periodic acid, prewarmed to room temperature, for 20 minutes. Rinse slides for five minutes in running tap water. Place slides in Schiff’s reagent, prewarmed to room temperature, for 20 minutes. Rinse slides in running tap water for 12 minutes. Counterstain for ten minutes in Mayer’s hematoxylin. Rinse slides in running tap water for eight minutes. Dry in air and coverslip if desired.

**RESULTS AND COMMENTS**

PAS positivity is observed as a red color, the intensity of the color being roughly proportional to the quantity of glycogen present. The various types of PAS staining patterns are discussed elsewhere in this paper.
Acid Phosphatase Stain\textsuperscript{16, 18, 22, 29, 30, 35, 39}

**PRINCIPLE**

At an acid pH, acid phosphatase hydrolyzes a phosphate ester, naphthol AS-BI phosphoric acid yielding phosphate and naphthol AS-BI complex, which couples with the diazonium Fast Garnet GBC salt directly to yield insoluble brownish maroon granules or with hexazonium pararosaniline to yield insoluble red granules.

**REAGENT PREPARATION**


**PROCEDURE**

Fix two specimen and two control slides in citrate-acetone fixative for 30 seconds. Rinse slides in distilled water for 30 seconds. Dry in air.

Label two 100 ml beakers A and B and add:

<table>
<thead>
<tr>
<th></th>
<th>Beaker A</th>
<th>Beaker B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>46.0 ml</td>
<td>44.0 ml</td>
</tr>
<tr>
<td>Naphthol AS-BI Phosphoric Acid Solution</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Acetate Solution</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>L (+) Tartrate Solution</td>
<td>—</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Fast Garnet GBC Salt</td>
<td>15 mg</td>
<td>15 mg</td>
</tr>
</tbody>
</table>

Stir with magnetic stirrer for five minutes being careful not to contaminate any of the contents of beaker A with those of beaker B. Filter into separate Coplin jars labeled A and B. Place appropriate slides in Coplin jars A and B. Incubate at 37\(^\circ\), in the dark, in a hot air incubator for one hour. Remove slides and wash in distilled water for three minutes. (Keep A and B slides separate). Allow to air dry.

**RESULTS AND COMMENTS**

Acid phosphatase activity is observed as a brownish maroon pigment in the granules or cytoplasm of all cell lines. With tartrate inhibition, if the fraction 5 tartrate resistant isoenzyme is present, the acid phosphatase activity will be present almost exclusively in the lymphocytes.

Of the staining procedures outlined in this paper, the acid phosphatase procedure presents the most technical problems. Very small variations in techniques or reagents may result in no detectable activity. Hence, assessment of positive staining in the control slides must be made. Also, the resistance of the stains to fading is variable. Without known reason, the granules may fade rapidly, or may fade rapidly only if coverslipped. A method for scoring acid phosphatase activity similar to that of scoring alkaline phosphatase activity has been reported.\textsuperscript{29}

**Toluidine Blue Stain\textsuperscript{41}

**PRINCIPLE**

Toluidine blue stains heparin a dark reddish purple owing to the strong metachromatic effect of the sulfonic acid groups on heparin. Toluidine blue stain readily distinguishes the dark heparin containing granules of basophils and mast cells from the very dark toxic granulation sometimes observed in neutrophils, as well as the dark granules sometimes observed in promyelocytes and the dark granules in Alder-Reilly anomaly.

**REAGENT PREPARATION**

*Toluidine Blue 0 Stain Reagent. Dissolve 1 g of toluidine blue 0 in 100 ml of methanol. (Stable for one year at 25\(^\circ\)).*

**PROCEDURE**

Place specimen and control slides on a staining bridge. Flood each slide with toluidine blue 0 stain reagent. Allow to stand for five minutes. Rinse with tap water for one minute. Dry in air and coverslip if desired.

**RESULTS AND COMMENTS**

Positive toluidine blue staining for heparin is observed as dark-purple-red stain in the granules of basophils and mast cells. Fixation and staining are accomplished in a single step.

**Alkaline Phosphatase Stain\textsuperscript{1, 9, 13, 18, 19, 30, 35, 38}

**PRINCIPLE**

At the pH of 8.6, alkaline phosphatase hydrolyzes naphthol AS-MX phosphate to phosphate and the naphthol AS-MX complex. The latter complex immediately couples to the diazonium Fast Blue RR Salt forming an insoluble blue complex at the site of activity. The intensity of staining is proportional to the enzymatic activity.

**REAGENT PREPARATION**

No prior reagent preparation is needed. For convenience, the naphthol AS-MX phosphate alkaline solution should be pipetted in two ml aliquots into glass test tubes, tightly covered and frozen so that only one tube is thawed for each run.

**PROCEDURE**

Pipet 2 ml of naphthol AS-MX phosphate alkaline solution into a Coplin jar. Add 48 ml of water. (Alternatively, wash a 2 ml aliquot of Naphthol AS-MX phosphate alkaline solution with 48 ml of water into
the Coplin jar). Add 25 mg of Fast Blue RR salt. Stir until dissolved.
Place specimen and control slides in solution and incubate for 30 minutes. (No fixing is necessary, discard incubation solution after use.) Remove slides, rinse with tap water for 30 seconds. Counterstain card incubation solution after use.) Remove slides, Rinse with tap water for one minute. Dry in air.

Rinse with Mayer’s hematoxylin for eight minutes. Until dissolved.

incubate for 30 minutes. (No fixing is necessary, dissolved.


References


Results and Comments

The leukocyte alkaline phosphatase activity is observed as a dark blue pigment in the granules of mature neutrophils. The cells are examined under oil.

In 1963, Kaplow\textsuperscript{19} proposed a rating scale for the LAP activity in mature neutrophilic cells. This scale is rated from 0 to 4 depending on the intensity of stain in the neutrophilic bands and polys. The total is obtained by grading 100 neutrophils and bands. Therefore, the total score has a possible range of 0 to 400. Basically, the scoring criteria set down by Kaplow for each cell and used in this laboratory are as follows:

0 = Colorless (No granules stained in cytoplasm).
1 = Diffuse, but slightly positive with occasional granules (1 to 25 percent of granules stained in cytoplasm). Faint to moderate intensity with colorless to pale blue cytoplasm.
2 = Diffusely positive, with moderate number of granules (26 to 50 percent of granules stained in cytoplasm). Moderate to strong intensity with colorless to pale blue cytoplasm.
3 = Strongly positive with numerous granules (51 to 75 percent of granules stained in cytoplasm). Strong to brilliant intensity with little cytoplasm visible.
4 = Very strongly positive, with very dark confluent granules (76 to 100 percent of granules in cytoplasm). Brilliant intensity with no cytoplasm visible.

Multiply the number of cells found in each category by its score. Add the scores together for the total leukocyte alkaline phosphatase score and report individual and total scores. The reference range is 13 to 130 in this laboratory. If greater than 90 percent of the cells score 0, even if the overall score is normal, the probability is great that the patient has chronic myelocytic leukemia or paroxysmal nocturnal hemoglobinemia.

The use of fresh non-anticoagulated specimens cannot be overstressed. The variability observed with storage and anticoagulants makes interpretations questionable. The blue pigment lasts indefinitely until immersion oil is put on the slide. Then, as with coverslipping, the intensity of stain may or may not fade.


