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Cytochemical Identification of Monocytes and Granulocytes

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ABSTRACT

Yam, L. T., Li, C. Y., and Crosby, W. H.: Cytochemical identification of monocytes and granulocytes. Amer. J. Clin. Path. 55: 283-290, 1971. Cytochemical methods for chloroacetate esterase, nonspecific esterase, peroxidase, and metachromasia were used to identify monocytes and neutrophilic, basophilic, or eosinophilic granulocytes. These methods are very simple, sensitive, and easily reproducible. Their reaction products are highly chromogenic and insoluble in most organic solvents. Any of these methods may be used independently or in combination with another cytochemical method to demonstrate a particular type of cell as desired. The specific aims and possible areas for clinical application of these cytochemical methods are discussed.

MORPHOLOGIC STUDY of Romanowsky-stained smears of blood and marrow forms the laboratory basis of clinical hematology. It is, however, more art than science, and its value rests heavily upon the skill of the observer. With the recent advances in therapy of blood diseases, accurate identification of cell types has become a practical necessity and is essential for meaningful evaluation and proper management. Investigation of cellular functions, interactions, immunity, and particularly, neoplasms, requires highly sophisticated methods to separate various types of cells for study. Reliability of these methods is often determined by the purity of cell preparations which, in turn, requires accurate identification of cell types.

In this paper we present several cytochemical methods which are useful in the accurate identification of monocytes and

neutrophilic, eosinophilic, and basophilic granulocytes.

Materials and Methods

Blood and bone marrow smears and lymph node and spleen aspirates and imprints were used. These smears can be stored unfixed at room temperature for at least 2 weeks without appreciable change in enzymic activity. A buffered formalin acetone mixture, pH about 6.6 (20 mg. Na₂HPO₄, 100 mg. KH₂PO₄, 30 ml. H₂O, 45 ml. acetone, and 25 ml. formalin) is the fixative for all the following cytochemical methods except the toluidine blue stain. The smears are fixed with this solution for 30 sec. at 4 to 10 C., washed by three changes of distilled water, and air-dried at room temperature for 10 to 30 min. before incubation.

A. Cytochemical Demonstration of Chloroacetate Esterase

1. Incubate fixed smears in a Columbia jar at room temperature for 10 min. in the following medium (without filtration): Phosphate buffer (M/15, pH 7.6), 9.5 ml.

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Freshly-prepared hexazotized new fuchsin, 0.05 ml.†

Naphthol AS-D chloroacetate,* 1 mg. per 0.5 ml. N-N dimethyl formamide

New fuchsin† solution was prepared according to the method of Stutte²⁰ in which 1 Gm. of new fuchsin was dissolved in 25 ml. of 2 N hydrochloric acid. Hexazotization was performed by allowing the new fuchsin solution to react with an equal volume of a fresh 4% sodium nitrite solution for 1 min. before use. The final pH of the incubating medium is about 7.4.

2. Wash with tap water, counterstain with 1% methyl green, buffered with 0.1 N sodium acetate to pH 4.2 for 1 to 2 min.

3. Wash with water, dry, mount in Permount, and examine. Enzymic activity is seen as bright red granules in the cytoplasm of the mast cells and neutrophilic granulocytes, including promyelocytes and many myeloblasts.

B. Cytochemical Demonstration of Nonspecific Esterase

1. Incubate fixed smears in a Columbia jar for 45 min. at room temperature in the following medium:

Phosphate buffer (M/15, pH 7.6), 8.9 ml. (4.5)

Hexazotized pararosanilin, 0.6 ml. (3.0 ml)

Alpha-naphthyl acetate,† 10 mg. per 0.5 ml. ethylene glycol monomethyl ether. (2.5 ml)

The pararosanilin solution is prepared according to the method of Barka and Anderson.² Hexazotization was made by mixing an equal volume of the pararosanilin solution and a fresh 4% sodium nitrite solution for 1 min. before use. The final pH of the incubating medium was adjusted with 1 N NaOH to pH 6.1 (range 5.8 to 6.5). The incubation medium was filtered before use.

* Sigma Chemical Co., St. Louis, Missouri 63118.

† National Aniline Division, New York, New York 10006 (C.I.#42520).

‡ Sigma Chemical Co., St. Louis, Missouri 63118.

2. Wash with water; counterstain with 1% methyl green for 1 to 2 min.

3. Wash with water, dry, mount with Permount, and examine. Enzymic activity is seen as dark red granules, mainly in the cytoplasm of monocytes, histiocytes, and megakaryocytes.

C. Combined Method for Chloroacetate Esterase and Nonspecific Esterases

1. Stain smears for nonspecific esterase at room temperature for 30 to 45 min. as in method B.

2. Wash with three changes of distilled water, incubate smears in the following medium for chloroacetate esterase at room temperature for 10 min. This medium should be filtered before use.

Phosphate buffer (M/15, pH 7.4), 9.5 ml.

Naphthol AS-D chloroacetate, 1 mg. per

0.5 ml. N-N dimethyl formamide

Fast blue BBN § 5 mg.

3. Wash with water; counterstain with 1% methyl green for 1 to 2 min.

4. Wash, dry, mount in Permount, and examine. Chloroacetate esterase activity is indicated by discrete blue granules in the cytoplasm of the granulocytes and nonspecific esterase is indicated by dark red granules in the monocytes.

D. Peroxidase

Peroxidase reaction is performed according to the method of Kaplow.⁷¶ Enzymic activity in the eosinophils is selectively demonstrated by incubating fixed smears for 30 sec. in 10 ml. of Kaplow's medium containing 4.9 mg. of sodium cyanide (10^{-2} M), with the final pH adjusted to 6.1 with 1 N HCl. When counterstaining is desirable, smears are stained by a 1% neu-

§ Cyclo Chemical Corporation, Los Angeles, California 90001.

¶ Benzidine hydrochloride (highly purified) used in this reaction is purchased from Hartman-Leddon Co. (Cat. #5448), Philadelphia, Penna.

tral red in phosphate buffer (pH 4.5 to 5.0) for 1 min.

E. Combined Method for Chloroacetate Esterase and Peroxidase

1. Stain smears for chloroacetate esterase as in method A.

2. Wash with water and counterstain with methyl green for 1 to 2 min.

3. Wash with distilled water and stain for peroxidase as in method D.

4. Wash with water, dry, mount with Permount, and examine.

Neutrophilic granulocytes, including myeloblasts and promyelocytes, contain both blue and red granules, indicating activities of both enzymes. Eosinophils and monocytes contain blue granules only, indicating peroxidase activity. However, when cyanide is present in the incubation medium for peroxidase, only the eosinophils are positive.

F. Combined Method for Nonspecific Esterases and Peroxidase

1. Stain smears for nonspecific esterases at room temperature for 30 min. as in method B.

2. Wash with water and counterstain with 1% methyl green for 1 to 2 min.

3. Wash with water and stain for peroxidase as in method D for 30 sec.

4. Wash with water, dry, mount in Permout, and examine. Peroxidase activity is seen as blue granules in the cytoplasm of the granulocytes and some monocytes, whereas nonspecific esterase activity is seen as dark red granules in the monocytes.

Basophils show very weak or negative activity for peroxidase, chloroacetate esterase, and nonspecific esterase, but they contain metachromatic granules. The following method of toluidine blue staining is most satisfactory for the demonstration of metachromatic granules in the basophils.

G. Toluidine Blue Stain

1. Smears are fixed in Mota's fixative¹⁸ for 1 min at room temperature. The fixative is prepared by mixing 1 Gm. lead subacetate, 50 ml. ethanol, 50 ml. distilled water, and 0.5 ml. glacial acetic acid. This fixative is stable at room temperature for at least 1 month.

2. Wash with distilled water and stain for 5 min. in 0.1% toluidine blue 0 in 30% ethanol.

3. Wash with water, dry, mount with Permout, and examine.

The smear can be further stained for peroxidase or cyanide-resistant peroxidase as in method D.

Specificity of these methods is examined by boiling the fixed smears for 2 min. or by omitting either the substrates or the couplers. The effects of sodium ethylenediamine tetraacetate, heparin, and citrate were tested. None of these three anticoagulants was found to be inhibitory to any of these enzymes.

Other methods for chloroacetate esterase,^{9,10,12,17} nonspecific esterases,^{3-5,11,17,18,22} and peroxidases^{6,7,15,16,19,21,23} were also examined, and the results compared with those of the methods used here. The degrees of purity of lymphocytic preparations resulting from several methods^{14,24} were also examined by the above-mentioned cytochemical methods.

Results

The activities of chloroacetate esterase, nonspecific esterase, and peroxidase in blood cells, as well as the results of combined staining methods, are listed in Tables 1 and 2. The granulocytes, including promyelocytes and many myeloblasts, showed very strong activity for chloroacetate esterase, whereas the monocytes and basophils showed little or no activity.

The eosinophils, lymphocytes, erythroblasts, plasma cells, and megakaryocytes contained no chloroacetate esterase activity (Figs. 1, 4, 6, 10, and 11).

Nonspecific esterase activity is very strong in the monocytes, histiocytes, and megakaryocytes. It is very weak in the granulocytes and the lymphocytes (Figs. 2, 4, 6, and 8). Occasionally, a few eosinophilic myelocytes may show more activity than the ordinary granulocytes, but these cells can be identified easily by their large granules or by the peroxidase reaction in the presence of cyanide.

The eosinophils and neutrophilic granulocytes, including promyelocytes and many of the myeloblasts, are strongly positive for peroxidase. The monocytes are moderately peroxidase positive, but occasionally strongly so (Figs. 9 and 11). There is no demonstrable qualitative difference between enzyme activities in the monocytes and the neutrophilic granulocytes, as indicated by the inhibitor studies using azide, bivalent metal salts, tartrate, and fluoride. However, as shown by Archer,¹ part of the peroxidase activity in the eosinophils is cyanide-resistant. After the addition of

10⁻² M cyanide to the incubation medium for peroxidase, only the eosinophils are stained blue-green, while all the other cells are colorless. This contrast can be further augmented by staining the other granulocytes in red with the hexazotized new fuchsin method for chloroacetate esterase (Fig. 10). If the smear is stained for chloroacetate esterase and peroxidase in the absence of cyanide, many of the granulocytes are shown to have both these enzymes in the same cell, whereas others may stain with one of these enzymes only (Fig. 11).

The toluidine blue method is specific for the demonstration of metachromasia in basophils and their precursors as well as in mast cells. Mota's fixative, using lead subacetate, preserves the metachromatic granules in these cells better than methanol, ethanol, or formalin vapor (Fig. 12).

Discussion

Our methods for chloroacetate esterase and for nonspecific esterase provide some advantages over previously reported methods. They are simple, very sensitive, and easily reproducible, and enzyme localiza-

Table 1. Results of Nonspecific Esterase, Chloroacetate Esterase, Peroxidase, and Metachromasia Stains of Blood and Marrow Cells

	Nonspecific Esterase	Chloroacetate Esterase	Peroxidase		Metachromasia
			Without Cyanide	With Cyanide	
Myeloblasts	0-±	0-+++	0-+++	0	0
Promyelocytes	0-±	++++	++++	0	0
Neutrophilic granulocytes	0-±	++++	++++	0	0
Eosinophilic granulocytes	0-+	0	++++	+++	0
Basophilic granulocytes	0	0-+	0-+++	0	++-+++
Megakaryocytes	++++	0	0	0	0
Erythroblasts	0-+	0	0	0	0
Lymphocytes	0-+	0	0	0	0
Plasma cells	0-++	0	0	0	0
Monocytes	++++	0-+	0-+++	0	0
Reticulum cells (histiocytes)	++++	0-+	0	0	0
Mast cells	?	++++	0	0	++++

Key: 0=no activity, ±=questionable, +=weak, ?=unknown, ++=moderate, +++=strong, ++++=very strong activity.

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Table 2. Cell Specificity and Possible Clinical Applications of the Various Cytochemical Reactions

Cytochemical Reactions	Specific Aims	Clinical Application
Nonspecific esterase	Monocytes, histiocytes	Marker for monocytes, histiocytes, acute monocytic leukemia
Chloroacetate esterase	Neutrophilic granulocytes	Marker for granulocytes, acute granulocytic leukemia
Peroxidase	Granulocytes, monocytes	Acute leukemia
Cyanide-resistant peroxidase	Eosinophils	Marker for eosinophils
Nonspecific esterase with chloroacetate esterase	Monocytes, neutrophilic granulocytes	Marker for monocytes, granulocytes, acute leukemia
Nonspecific esterase with peroxidase*	Monocytes, granulocytes	Acute leukemia
Chloroacetate esterase with peroxidase*	Granulocytes, monocytes	Increased sensitivity for acute granulocytic leukemia
Metachromasia	Basophils, mast cells	Marker for basophils and mast cells

* Peroxidase activity weakens 24 hours after mounting in Permout.

tion is precise. The reaction products are highly chromogenic and insoluble in most organic solvents. Further staining procedures for other enzymes and various counterstains can be used if needed. Smears stained by these methods can be mounted in Permout and kept permanently.

Several groups of workers have used the nonspecific esterase as the marker enzyme for monocytes.^{5, 18} In their methods, the myelocytes exhibited fairly strong activity but could be differentiated from the monocytes by having some activity in the presence of fluoride. In our method using alpha naphthyl acetate and hexazotized pararosanilin, enzymic activity in the granulocytes is diffuse and very weak, whereas the activity in the monocytes is granular and very strong. The contrast between these two is so marked that it is not necessary to use fluoride for further differentiation. Our method is very useful in identification of monocytes and histiocytes in smears and in assessing the purity of lymphocytic preparation (Figs. 7 and 8).

The combined method for nonspecific es-

terase and chloroacetate esterase provides an objective and accurate means of demonstrating monocytes and granulocytes simultaneously in many cytologic preparations. In the acute leukemias, this combined cytochemical method is superior to the Romanowsky stains in cell identification and may aid in the differential diagnosis of acute monocytic or granulocytic leukemias (Figs. 3 to 6). In a rare case when the myeloblasts show little or no activity for chloroacetate esterase, the nonspecific esterase-peroxidase method can be used instead.

Monocytes and granulocytes both are positive for peroxidase. It is frequently felt that these two types of cells can be differentiated by the peroxidase reaction because the activity of this enzyme in the monocytes is weaker than that found in the granulocytes; however, some monocytes may have very strong peroxidase activity, mimicking that of myelocytes (Fig. 9). This quantitative difference between enzymic activities in the monocytes and the granulocytes may become negligible when a method as sensitive as Kaplow's is used. In this regard, the

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chloroacetate esterase reaction is more specific for the granulocytes than the peroxidase reaction.

Several methods for chloroacetate esterase^{9, 10, 12, 17} and peroxidase^{6, 7, 15, 16, 19, 21, 23} were examined to determine their ability to stain the myeloblasts in blood from patients with acute leukemia. Kaplow's method⁷ was the most sensitive for peroxidase and the hexazotized new fuchsin method was the most sensitive for chloroacetate esterase. However, the blasts from some known cases of granulocytic leukemia have failed to stain with either the chloroacetate esterase method or Kaplow's method.⁸ The combined technic for chloroacetate esterase and peroxidase may prove to be more sensitive for the identification of myeloblasts in acute granulocytic leukemia than either method alone (Fig. 11).

It is generally accepted that the neutrophilic, eosinophilic, and basophilic granulocytes are derived from a common myeloblast and promyelocytes. If this is true, then traceable connections of enzyme systems or intermediate metabolic products should be found in these cells. However, when the granulocytes were examined for peroxidase (cyanide-resistant or cyanide-sensitive), chloroacetate esterase and meta-

chromasia, only the neutrophilic, but not the eosinophilic or basophilic, granulocytes showed traceable connections with myeloblasts and promyelocytes (Table 1). The eosinophilic myelocyte and its descendants differ from these myeloblasts and promyelocytes by having cyanide-resistant peroxidase and lacking chloroacetate esterase. The basophilic myelocyte and its descendants differ from the myeloblasts and the promyelocytes in having metachromatic granules and only very weak activity of chloroacetate esterase and peroxidase. These findings suggest that the eosinophilic and the basophilic granulocytes may be derived from some very rare myeloblasts or promyelocytes that are different from the mother cell of the neutrophilic granulocytes. Such young cells with cyanide-resistant peroxidase activity or with metachromatic granules are indeed present in bone marrow smears from patients with or without leukemia. They are very rare in the marrows of nonleukemic subjects and are generally missed unless the specially stained smears are carefully examined. However, in smears from several patients with chronic granulocytic leukemia with eosinophilia or basophilia these young cells have been found more readily.

FIG. 1. Chloroacetate esterase reaction. Six neutrophilic granulocytes are strongly positive, whereas one eosinophil and one normoblast are negative. New fuchsin method. $\times 850$.

FIG. 2. Nonspecific esterase reaction. Two monocytes are strongly reactive, whereas three granulocytes are either weak or nonreactive. $\times 850$.

FIG. 3. Blood from a patient with acute myelomonocytic leukemia, showing blasts and monocytes. Wright Giemsa stain. $\times 850$.

FIG. 4. Blood from patient in Figure 3, showing monocytes (red) and granulocytes (blue). Double staining method for nonspecific esterase and chloroacetate esterase. $\times 850$.

FIG. 5. Marrow smears from a patient with acute leukemia. Wright Giemsa stain. $\times 850$.

FIG. 6. Marrow smear from the patient in Figure 5, showing predominance of monocytes and a few granulocytes, suggesting monocytic leukemia of the Schilling type. Double staining method for nonspecific esterases and chloroacetate esterase. $\times 340$.

FIG. 7. Lymphocytic preparations from the human spleen. Wright Giemsa stain. $\times 170$.

FIG. 8. Same as in Figure 7, showing four monocytes. Nonspecific esterase reaction. $\times 170$.

FIG. 9. Note that the monocytes have enzymic activity as strong as that of the granulocytes. Peroxidase reaction, showing two monocytes (arrows) and two myeloblasts. $\times 850$.

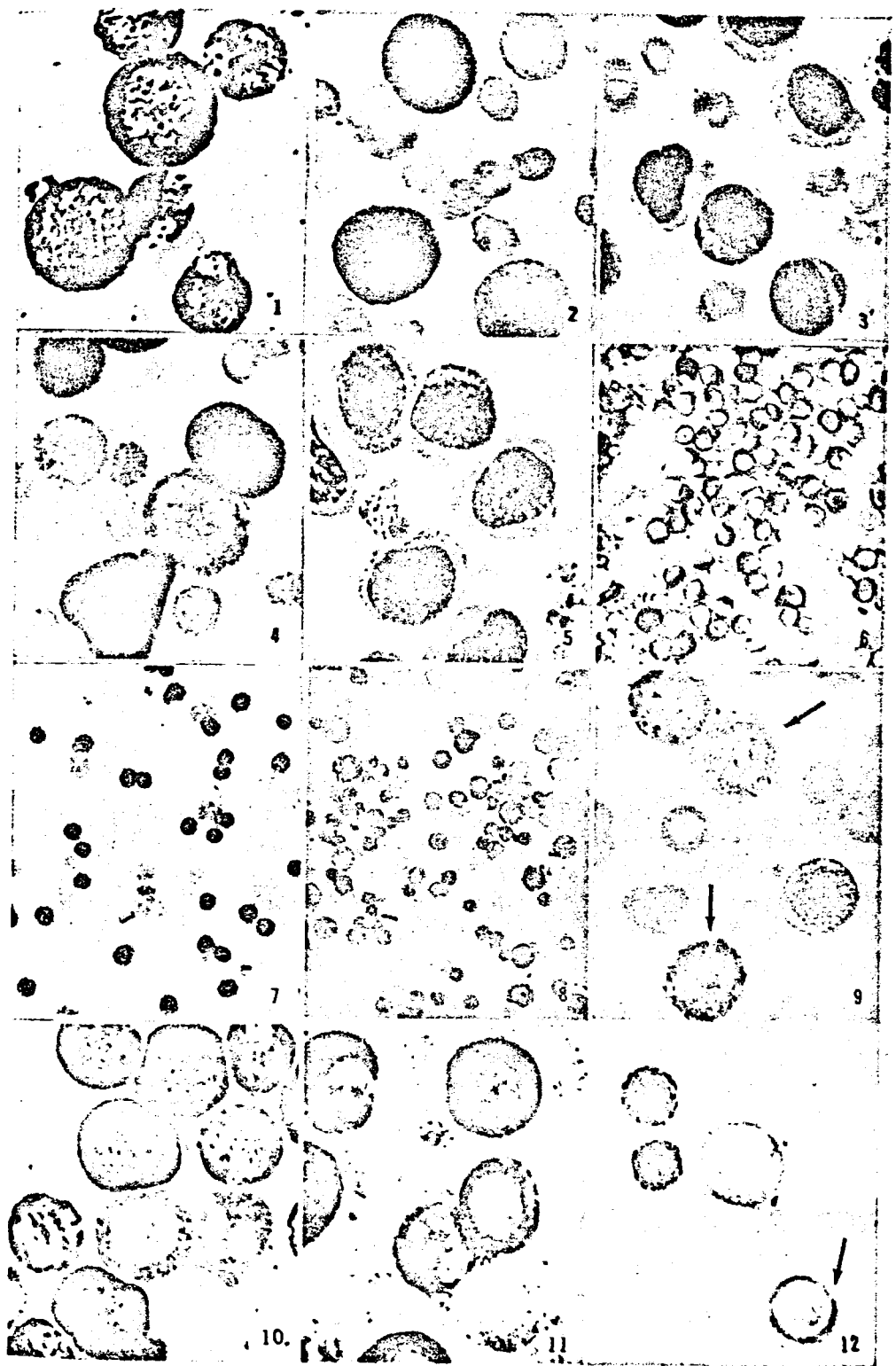
FIG. 10. Selective demonstration of eosinophilic (blue) and neutrophilic (red) granulocytes. Chloroacetate esterase-peroxidase with cyanide method. $\times 850$.

FIG. 11. Combined method for peroxidase and chloroacetate esterase, demonstrating that many granulocytes have both enzymes in the same cell, whereas one has only chloroacetate esterase activity (red) and three have only peroxidase activity (blue). This combined method increases the sensitivity for the diagnosis of acute granulocytic leukemia. $\times 850$.

FIG. 12. Selective demonstration of basophils, showing two mature basophils exhibiting many metachromatic granules, but one immature cell also has a few metachromatic granules (arrow). $\times 850$.

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