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1967

CYTOCHEMICAL STUDIES OF
ACUTE LEUKEMIAS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * *

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The morphological identification of the blood and bone marrow cells of normal individuals and patients with leukemia using Romanowsky stains is well recognized. This staining method has been widely used for the differential diagnoses of hematological disorders. However, in leukemia using only morphological criteria, distinction between the types of stem cells has been difficult.

Recent studies have applied cytochemical methods extensively in studying acute leukemias. Most investigators, however, have employed only one or two cytochemical reactions.^{18,37,38,90,101,103,106,179} Variations in glycogen^{2,3,37,38,90,124} and lipids^{87,101,106,145,179} and the differences in the reactivity of alkaline phosphatase,^{7,87,90,102,176,200} acid phosphatase,^{70,103,122} nonspecific esterase,^{18,87,203} sulfhydryls,^{26,27,43} aminopeptidase,^{6,9} phosphorylase,^{77,87} and lactic dehydrogenase^{87,162} have been described in the blood and bone marrow cells

of normal individuals and patients with acute leukemias. Such observations suggest that cytochemical studies do provide additional criteria for the differentiation and classification of the acute leukemias.

The cytological and cytochemical methods used in the present study have been directed toward the morphological, chemical, and enzymatic differentiation and the subsequent classification of the primitive cells of the acute leukemias. In combining cytochemical and morphological analysis, it should be possible to correlate alterations in cellular structure with differences in chemical composition. Recent evidence has indicated that the major defect in the acute leukemias is the impairment of cellular differentiation and maturation.^{62,64,65,177} Therefore, studies directed toward obtaining cytochemical profiles of the primitive cells may be of valuable assistance in determining the cause or causes underlying the alterations in cellular development.

MATERIALS AND METHODS

Blood and bone marrow films were obtained from 71 patients with acute leukemia; 29 with acute lymphocytic

leukemia (ALL); 23 with acute monocytic leukemia (AML); 19 with acute granulocytic leukemia (AGL) including 5 patients in the acute phase of chronic granulocytic leukemia. When the blood and bone marrow films were procured, 17 of the patients with ALL had been treated (5 with 6-mercaptopurine, 4 with prednisone, 4 with cytosine, 3 with amethopterin, 1 with amethopterin and hydrocortisone) and 12 had had no therapy; 12 of the patients with AML had been treated (9 with 6-mercaptopurine, 2 with hydrocortisone, 1 with prednisone and 6-mercaptopurine) and 11 were untreated; 12 of the patients with AGL had been treated (6 with myleran, 3 with hydrocortisone and 6-mercaptopurine, 2 with 6-mercaptopurine, 1 with hydrocortisone) and 7 were untreated. The diagnoses of the type of acute leukemias were made at Simpson Memorial Institute and the Hematological Section of the Department of Pediatrics, University of Michigan.

Controls consisted of blood films from 12 healthy laboratory workers, bone marrows from 4 healthy adults, and 3 patients with non-hematological diseases.

Included in the controls were 6 patients with idiopathic thrombocytopenic purpura (4 were untreated and 2 were in remission). All patients used as controls had normal leukocyte counts and differentials.

Morphological evaluation of the blood and bone marrow films of the patients with acute leukemia and the controls was accomplished by examining films stained with Wright's stain. Cytochemical characteristics were amassed by using seven cytochemical reactions: periodic acid-Schiff reaction, Sudan black B reaction, and reactions for acid phosphatase, alkaline phosphatase, nonspecific esterase, phosphorylase, and lactic dehydrogenase.

Periodic Acid-Schiff Reaction. The periodic acid-Schiff reaction with salivary amylase digestion was used for the identification of glycogen and glycoprotein. Formalin vapor was used as the fixative.

Sudan Black B Reaction. The method of Sheehan and Storey (1947) was employed for the demonstration of lipids. Air dried films were fixed in formalin vapor and immersed in the Sudan black B staining solution for one hour at room temperature.

Acid Phosphatase Reaction. Acid phosphatase activity was demonstrated by the naphthol AS-BI phosphate hexazonium pararosanilin method described by Goldberg and Barka (1962). Formol vapor, formol-calcium fixation was employed and the films were incubated in the substrate solution for one and one-half hours at thirty-seven degrees Centigrade.

Alkaline Phosphatase Reaction. The naphthol AS-MX phosphate-Fast blue RR method of Ackerman (1962) was employed for the localization of alkaline phosphatase activity. The formol-methanol fixed films were incubated for forty-five minutes at room temperature.

Nonspecific Esterase Reaction. Naphthol AS acetate-Fast blue BB method of Wachstein and Wolf (1958) was used to demonstrate nonspecific esterase activity. The films were fixed in formalin vapor and incubated for ninety minutes at room temperature.

Phosphorylase Reaction. The phosphorylase method of Takeuchi and Kinoshita (1956) as modified by Ern and Palkama (1961) was employed in this study. However, a higher concentration of glucose-1-phosphate (150 mg/ml)

and glycogen (10 mg/ml) was used in the substrate solution. These changes appeared to improve the sensitivity of the phosphorylase reaction.

Lactic Dehydrogenase Reaction. The Quaglino and Hayhoe (1960) procedure was employed for the localization of lactic dehydrogenase activity. Acetone-fixed films were incubated in the substrate solution with nitro BT as the tetrazolium salt for one to two hours at thirty-seven degrees Centigrade.

Semiquantitative Scoring Method. A semiquantitative scoring method¹⁶ was used for estimating the intensity of the cytochemical enzyme reactions of the primitive cells of patients with acute leukemias and blood and bone marrow cells of normal controls. The rating of individual cells was as follows:

- 0 Colorless
- 1+ Diffuse staining or fine granules, or both;
5 to 15 granules.
- 2+ Moderate; moderately coarse granules;
16 to 30 granules.
- 3+ Strong; coarse granules; 31 to 45 granules.

4+ Intense; densely packed or very coarse granules and/or clumps of reactive material; 45 or more granules.

Using this scoring procedure, the highest mean score possible would be four hundred. To obtain a total score, one hundred primitive cells of patients with acute leukemias were counted for each reaction. Lymphocytes, monocytes, and myeloblasts of normal individuals were used as appropriate controls, and one hundred cells of each type were counted in order to obtain total scores for each reaction. Alkaline phosphatase activity was scored by counting one hundred of the mature neutrophils of the blood and bone marrows of patients with acute leukemias and normal controls.

OBSERVATIONS

The morphological characteristics of the cells in acute leukemias as observed in Wright's stain are detailed in Table 1. In general, the characteristics conform to criteria used at the University of Michigan and to other observations^{46,87,209} employed in the identification of the various cell types and forms of acute leukemias.

Periodic Acid-Schiff Reaction. An assessment of the scores of the periodic acid-Schiff reaction (PAS) in ALL varies between 62 and 214 with a mean of 134 (Table 2; Chart 1). Although individual cells exhibit considerable variation in PAS reactivity, the majority of lymphoblasts observed in patients with ALL possess a moderate (++) to strong (+++) reactivity. In contrast, normal lymphocytes, used as a comparison since lymphoblasts are not present in normal blood and bone marrow, exhibit a considerably lower mean score of 39 with a range between 26 and 62 (Table 2; Chart 2). Cytologically, the PAS reactive material in lymphoblasts of ALL has a peripheral cytoplasmic distribution appearing as fine to coarse particulate granules with coarse clumps of positive material frequently seen in some of the cells (Plate II). In lymphocytes of normal blood, the pattern of the PAS reaction is similar (Plate III) to lymphoblasts of ALL, although coarse clumps of positive material are not observed.

The PAS scores of the monoblasts observed in patients with AML vary between 48 and 121 with a mean

of 88 (Table 2; Chart 1). Many monoblasts show only a diffuse cytoplasmic coloration; other cells have fine granules (+, ++), and some cells possess many coarse granules (Plate II) with moderate to strong coloration (++, +++). The monocytes in normal blood, used as a standard for comparison of the monoblasts in AML, have similar PAS scores, ranging between 47 and 105 with a mean of 74 (Table 2; Chart 2), and a similar distribution of PAS reactive material.

The PAS scores of the myeloblasts observed in patients with AGL range between 28 and 102 with a mean of 53 (Table 2; Chart 1) and are slightly higher than in the myeloblasts observed in the bone marrow of normal individuals, which range between 21 and 67 with a mean of 35 (Table 2; Chart 2). Many of the myeloblasts of AGL are nonreactive or exhibit a diffuse staining of their cytoplasm (Plate II). Occasionally, particulate glycogen granules can be seen. Myeloblasts from normal individuals exhibit PAS reaction patterns similar to the myeloblasts of AGL, although particulate glycogen granules are not observed.

Sudan Black B Reaction. Sudan black B reaction shows similar reactions in the primitive cells of the acute leukemias and normal blood and bone marrow cells. Lymphoblasts of ALL and the lymphocytes of normal blood are often negative or possess only a few sudanophilic granules (Plate III); monoblasts of AML and monocytes of normal blood demonstrate many small sudanophilic granules scattered throughout the cells (Plate III); myeloblasts of AGL show slightly greater sudanophilic reactions than the myeloblasts of normal blood which have faint reaction patterns. In normal granulocytes, Sudan black B staining appears to increase with the maturation of the cells. Semiquantitative scoring was not done on the Sudan black B reactions.

Acid Phosphatase Reaction. The acid phosphatase scores of the lymphoblasts of ALL range between 60 and 131 with a mean of 90 (Table 2; Chart 3) being similar to the acid phosphatase scores of lymphocytes of normal blood which vary between 58 and 120 with a mean of 94 (Table 2; Chart 4). In general, the acid phosphatase activity appears as weak to moderate (+, ++).

Reactivity in the lymphoblasts of ALL localizes in one or more areas near the periphery of the cytoplasm with the most intense reaction appearing near the nuclear hof (Plate IV). In lymphocytes of normal blood the pattern is similar to the lymphoblasts with small areas of reactivity localizing near the periphery of the cells (Plate V); an occasional lymphocyte exhibits a more intense perinuclear activity.

The level of acid phosphatase scores of monoblasts of AML appears somewhat higher than that of the lymphoblasts of ALL. In monoblasts of AML, the acid phosphatase scores vary between 93 and 155 with a mean of 128, being similar to the acid phosphatase scores of monocytes of normal blood which have a range between 108 and 150 with a mean of 132 (Table 2; Charts 3,4). The monoblasts of AML exhibit varied reactivity from moderate to strong (++, +++). The acid phosphatase activity, localizing primarily in small and large granules, is most intense at the nuclear hof but with some areas of activity appearing near the periphery of the cell (Plate IV). Monocytes of normal blood, like monoblasts of AML,

exhibit varied degrees of reactivity from moderate to strong (++, +++) with the localization most intense in the perinuclear cytoplasm. An occasional monocyte of normal blood shows intense cytoplasmic activity (Plate V).

The acid phosphatase scores of the myeloblasts of AGL show a variation between 96 and 143 with a mean of 120. The acid phosphatase scores of the myeloblasts of AGL were judged higher than the acid phosphatase scores of myeloblasts of normal bone marrow which range between 68 and 99 with a mean of 86 (Table 2; Charts 3,4). The myeloblasts of AGL exhibit moderate to strong activity (++, +++) with more granules depositing in a perinuclear position (Plate IV). Myeloblasts of normal bone marrow show smaller areas of reactivity with one to several small clusters of granules localizing near the nuclear hof (Plate V).

Alkaline Phosphatase Reaction. The primitive cells in leukemia, like the normal precursor cells, are negative for alkaline phosphatase activity. However, since the alkaline phosphatase activity is demonstrable in the

mature neutrophils of the patients with acute leukemia, the neutrophils can be used in assessing levels of alkaline phosphatase activity. The results show that the scores of the neutrophils in ALL are the highest with a range between 100 and 272 and a mean of 180. The scores of the neutrophils in AML are intermediate with a range between 35 and 157 and a mean of 91. In AGL the neutrophils show scores, judged the lowest, varying between 9 and 44 with a mean of 25 (Table 3; Chart 5). Many of the neutrophils of AGL appear to have alterations in the size and number of the specific granules. The scores of the neutrophils of normal blood are also intermediate ranging between 60 and 156 with a mean of 82 (Table 3; Chart 5).

Nonspecific Esterase Reaction. The scores of the nonspecific esterase activity of the lymphoblasts of ALL range between 38 and 101 with a mean of 71 being similar to the lymphocytes of normal blood which vary between 42 and 95 with a mean of 68 (Table 2; Charts 6,7). The lymphoblasts of ALL have a weak to moderate reactivity (+, ++) with fine to coarse granules localizing

primarily in the perinuclear cytoplasm (Plate V). Lymphocytes of normal blood show similar reactivity (+, ++) with a peripheral cytoplasmic distribution of fine to coarse granules.

The monoblasts of AML and the monocytes of normal blood have higher nonspecific esterase scores than the lymphoblasts of ALL and the lymphocytes of normal blood. The nonspecific esterase scores of the monoblasts of AML range between 120 and 185 with a mean of 144, being similar to the scores of the monocytes of normal blood which vary between 105 and 149 with a mean of 140 (Table 2; Charts 6,7). The monoblasts of AML and the monocytes of normal blood exhibit strong to intense reactivity (++, +++) with heavier and coarser granules depositing randomly in the cytoplasm (Plate VI).

An assessment of the nonspecific esterase scores of the myeloblasts of AGL shows the level of scores to range between 49 and 88 with a mean of 75 (Table 2; Chart 6). The myeloblasts of normal bone marrow have scores between 63 and 87 with a mean of 73 (Table 2; Chart 7). These scores were judged lower than the

nonspecific esterase scores of the monoblasts of AML and the monocytes of normal blood. The reactivity of the myeloblasts of AGL and the myeloblasts of normal blood appears as a scattering of fine granules (+, ++) throughout the cytoplasm with most intense localization at the nuclear hof (Plates VI,VII).

Phosphorylase Reaction. In general, the degree of phosphorylase activity and its intracellular localization in the primitive cells of the acute leukemias and of normal blood and bone marrow cells corresponds closely with the cytoplasmic distribution and the relative amount of preformed glycogen demonstrated by the PAS reaction (Table 2; Charts 8,9; Plates VII,VIII). Occasionally, individual blast cells from the acute leukemias show a more intense phosphorylase reaction than would be expected from their PAS reactivity.

Lactic Dehydrogenase Reaction. Lactic dehydrogenase scores in the lymphoblasts of ALL and the lymphocytes of normal blood are comparable. Scores in the lymphoblasts of ALL range between 86 and 157 with a mean of 117 and in the lymphocytes of normal blood

vary between 73 and 145 with a mean of 110 (Table 2; Charts 10,11). The activity of the lymphoblasts of ALL shows intensities of weak, moderate, and strong (+, ++, +++) and appears as fine and coarse granules localizing at the site of mitochondrial concentration with some granules depositing randomly throughout the cytoplasm (Plate VIII). Lymphocytes of normal blood exhibit a similar pattern (Plate IX).

The lactic dehydrogenase scores of the monoblasts of AML are similar to the lactic dehydrogenase scores of the lymphoblasts of ALL. Likewise, the lactic dehydrogenase scores of the monoblasts of AML and the monocytes of normal blood are comparable. Lactic dehydrogenase scores of monoblasts of AML range between 92 and 166 with a mean of 123 and in monocytes of normal blood range between 107 and 149 with a mean of 117 (Table 2; Charts 10,11). Cytologically, the reactivity in the monoblasts of AML and the monocytes of normal blood has the same intensity (+, ++, +++) with fine to coarse granules localizing primarily in the perinuclear cytoplasm (Plates VIII,IX).

The lactic dehydrogenase scores in the myeloblasts of AGL are comparable to the lactic dehydrogenase scores assessed for the lymphoblasts of ALL and the monoblasts of AML with the lactic dehydrogenase scores varying between 107 and 157 with a mean of 121. The myeloblasts of normal bone marrow were judged to have slightly lower lactic dehydrogenase scores ranging between 72 and 128 with a mean of 91 (Table 2; Charts 10,11). The activity of the myeloblasts of AGL is of greater intensity (++, +++) than that seen in the myeloblasts of normal blood in which the reactivity was weak to moderate (+, ++). Localization of the granules is most intense near the nuclear hof (Plate IX).

DISCUSSION

Cytochemical activity of the primitive cells of the acute leukemias has been shown by multiple cytochemical procedures. The cytochemical studies suggest that there are alterations in cellular differentiation which are reflected in the cytochemical profiles of the primitive cells. Variation in the level of scores and reaction patterns of the acid phosphatase activity, nonspecific

esterase activity, and the Sudan black and PAS reactions observed in the present study correlates closely with the cytoplasmic and granular development and maturation exhibited by the primitive cells.

Cytologically, acid phosphatase and nonspecific esterase activity localizes in both azurophilic and specific granules. Correlated electron microscopic cytochemical studies reveal that these enzymes reside in the azurophilic and specific granules of human and rabbit leukocytes.^{17,71,136,207} The cytological results show that the greatest acid phosphatase activity and non-specific esterase activity appears in the monoblasts of AML with moderate activity in the myeloblasts of AGL and the least activity in the lymphoblasts of ALL. These differences in enzymatic activity indicate the possibility that there are specific varieties of the primitive cells of the acute leukemias. Moreover, comparison of these results to the results of the normal controls suggests that altered enzyme patterns or impaired cytoplasmic differentiation may occur in association with the formation of azurophilic granules at an earlier time

in cellular development. In comparing the reactions of the monoblasts of AML with the monocytes of normal blood or the reactions of the lymphoblasts of ALL with the lymphocytes of normal blood, the intensities of acid phosphatase and nonspecific esterase activities are similar indicating that the monoblasts of AML and the lymphoblasts of ALL may reflect altered granular development and/or cytochemical differentiation. The increased acid phosphatase activity of the myeloblasts of AGL as compared to the acid phosphatase activity of the myeloblasts of normal blood shows that similar alterations in cellular development may occur in the myeloblasts of AGL.

Recent cytochemical studies and electron microscopic cytochemical evidence indicates that lipids localize in the mitochondria and the cytoplasmic granules of the leukocytes.^{10,18,87,91} The Sudan black reaction demonstrates that lipids increase with the maturation of the normal granulocytes correlating with the increase in number of the specific granules.^{19,87,179} The myeloblasts of AGL which show reaction patterns similar to normal myelocytes suggest the presence of numerous

cytoplasmic granules at an earlier time in maturation. The monoblasts of AML with numerous Sudan black B reactive granules indicate that many azurophilic granules and/or mitochondria are present. The lymphoblasts of ALL exhibit only minimal Sudan black reactions and closely resemble the reaction seen in normal lymphocytes. Such observations suggest the possibility of aberrant cellular differentiation of the granules and/or mitochondria of the developing primitive cells.

The higher PAS scores of the lymphoblasts of ALL as compared to the monoblasts of AML and the myeloblasts of AGL confirm the results of other investigations.^{87,161} The large granular clumps of glycogen often seen in lymphoblasts of ALL, the particulate glycogen observed in myeloblasts of AGL, and the more frequent occurrence of moderately coarse granules without diffuse coloration seen in monoblasts of AML may reflect an alteration in cellular metabolism since large aggregates of glycogen in normal lymphocytes or particulate glycogen in normal myeloblasts cannot be observed. The present study and other evidence^{87,163,214} suggests that the activity of

phosphorylase corresponds to the distribution of preformed glycogen. The increased levels of phosphorylase activity that were found parallel closely the increased levels of preformed glycogen. These results tend to demonstrate that the increased levels of glycogen may result from the increase in the levels of phosphorylase activity.

The intracellular localization of lactic dehydrogenase (LDH) activity appears to reside in both the mitochondria and the cytoplasm of the leukocytes.^{10,20,87} Cytologically, the distribution of the LDH reactive granules in the primitive cells of the acute leukemias tends to follow the distribution of the mitochondria seen in normal leukocytes. Intragranular deposits of LDH activity are also noted. Recent biochemical observations^{34,54,60,61,156,158,177} indicate that LDH activity in various tissues exists in multiple molecular forms with five isoenzyme patterns. The isoenzyme patterns of the acute leukemias are identical with a predominance of LDH-3 activity which is similar to patterns obtained from fetal tissues^{156,177} and immature granulocytes.^{53,54}

The cytochemical levels of LDH activity of the primitive cells were judged to be similar and agree with the biochemical results suggesting that at a cellular level the LDH activity in the primitive cells is comparable in ALL, AML, and AGL. The levels of lactic dehydrogenase scores of the primitive cells of the acute leukemias are slightly higher than comparable normal controls and agree with biochemical investigations which indicate that the concentration of the dehydrogenases is increased in leukemic leukocytes.²⁹

The primitive cells of the acute leukemias are non-reactive to the alkaline phosphatase reaction. However, in assessing the scores of the neutrophils of the acute leukemias, there is sufficient variation in the scores to aid in a differential diagnosis of the acute leukemias. The current results agree with other evidence^{85,87} which show high alkaline phosphatase scores in ALL, intermediate scores in AML, and low scores in AGL. Cytochemistry, at both the light and the electron microscopic levels, reveals that alkaline phosphatase activity localizes in the specific granules of

the mature neutrophils of the granulocytes.^{10,17,91,136,207}
The altered levels of alkaline phosphatase activity observed in the mature neutrophils of AGL which show variations in the size and number of the specific granules may be compatible with a qualitative and quantitative difference in the specific granules that may have occurred during differentiation. The higher levels of alkaline phosphatase activity seen in the mature neutrophils of ALL and AML are not specifically attributable to the cellular lineage involved or an indication of granulocytic proliferation. It may indicate the normal response of the neutrophils with increased enzyme activity to infections and stress situations.^{87,193,197}

The primitive cells of the patients in the acute phase of chronic granulocytic leukemia do not demonstrate significantly different enzyme reaction patterns of the acid phosphatase, nonspecific esterase, and lactic dehydrogenase activities or the Sudan black B reaction as compared to the primitive cells of untreated patients with AGL.

Biochemical analysis of the primitive cells of the acute leukemias demonstrates low levels of alkaline phosphatase and acid phosphatase activity^{30,31,195,217} and normal levels of esterase and lipase activity.^{80,194} Investigations of glucose metabolism²⁹ indicate that leukemic leukocytes utilize the phosphogluconate pathway more than normal leukocytes. This is attributed to their deficiencies of hexokinase. Also, the same study revealed that the dehydrogenases were present in greater concentration than was necessary for their metabolic activity.²⁹ The low biochemical alkaline phosphatase activity is attributed to the low percentage of mature myeloid cells. Indications that there is more than one acid phosphatase²³ suggests the possibility that the biochemical methods may be demonstrating different enzyme reactions. In making chemical and cytochemical comparisons of abnormal cells in which various enzymes may vary or exhibit the same levels of activity, it may be advantageous to study intact cells which may possess important metabolic alterations that

may have resulted from aberrant organizations within the cell.²¹⁷

Recent evidence with the use of thymidine-labelled cells^{64,65} suggests that the bone marrow of patients with acute leukemias possesses two primitive cell populations, large primitive cells with proliferative ability and small primitive cells that are gradually losing the ability to proliferate. From this evidence it is postulated that the major cell defect in the acute leukemias is the inability for differentiation and maturation. Although the purpose of the present study was not to differentiate between two primitive cell populations of the bone marrows of the acute leukemias, an examination of the illustrations (Plate I, Figures 1,2,3,4; Plate II, Figures 6,7; Plate III, Figures 10,11; Plate IV, Figures 14,15; Plate VII, Figure 26; Plate VIII, Figures 31,32) tends to support cytochemically the postulation that dual primitive cell populations may exist. These observations show the possibility of intermediate cell forms between the larger and smaller primitive cells. Moreover, many of

the larger primitive cells demonstrate increased amounts of enzymatic reactive materials which suggests enhanced proliferative capability. Our cytochemical evidence, however, indicates that the primitive cells of the acute leukemias, which exhibit morphological and cytochemical variations, can be distinguished as specific types¹⁰ and are not modifications of a single precursor cell. The acid phosphate activity, the nonspecific esterase activity, and the Sudan black reaction show sufficient variation in their scores and reaction patterns to indicate the existence of several varieties of stem cells. The dissimilarity of the PAS reactive material and scores in the lymphoblasts of ALL, the monoblasts of AML, and the myeloblasts of AGL suggests the possibility of differences in these primitive cells.

SUMMARY

Blood and bone marrow films of seventy-one patients with acute leukemias and nineteen normal controls were evaluated morphologically by using Wright's stain and cytochemically by using seven cytochemical reactions: the periodic acid-Schiff reaction for the identification

of glycogen and glycoprotein, the Sudan black B reaction for lipids, the Goldberg and Barka method for acid phosphatase activity, naphthol AS-MX phosphate-Fast blue RR procedure for the alkaline phosphatase reaction, the naphthol AS acetate-Fast blue BB reaction for the demonstration of nonspecific esterase activity, the modified method of Eranko^{" "} and Palkama for phosphorylase reactivity, and the modified technique of Quaglino and Hayhoe for the localization of lactic dehydrogenase activity.

A semiquantitative scoring method was used for estimating the intensity of the cytochemical enzyme reactions of the primitive cells of patients with acute leukemias and blood and bone marrow cells of normal controls. Alkaline phosphatase activity was scored by counting mature neutrophils of the blood and bone marrow cells of patients with acute leukemias and normal controls.

Variations in the level of scores and reaction patterns of the acid phosphatase activity, nonspecific esterase reactivity, the alkaline phosphatase activity, and the Sudan black and PAS reactions provide additional

differential criteria for the separation and classification of the acute leukemias.

These cytochemical studies suggest that alterations may exist in cellular differentiation which are reflected in the cytochemical profiles of the primitive cells of acute leukemias. Altered enzyme patterns exhibited by the primitive cells correlate closely with the degree of cytoplasmic and granular development and/or maturation. Morphological and cytochemical variations indicate that the primitive cells of the acute leukemias can be identified and represent specific varieties of stem cells.

Table 1.--Cytological Characteristics in Wright's Stain of the Cells of
Blood and Bone Marrow of Patients with Acute Leukemias

Acute Lymphocytic Leukemia	Acute Monocytic Leukemia	Acute Granulocytic Leukemia
<p>Predominant cells are lymphoblasts. Round or oval nucleus. Coarse, deeply staining nuclear chromatin. Chromatin more compact around edges of nucleus and nucleoli. 1-2 nucleoli. Clear zone around nucleus. Cytoplasm basophilic; scant; contains no granules. Vacuolation can be marked. Mature lymphocytes usually present which contain a few azurophilic granules.</p>	<p>Predominant cells are promonocytes or monoblasts. Nuclei irregular in shape; often appear twisted and folded. Fine, reticular chromatin. 1-2 large nucleoli. Cytoplasm abundant; grey-blue with fine, reddish-lilac azurophilic granules. Cell boundaries irregular. Extensive vacuolation.</p>	<p>Predominant cells are myeloblasts. Round or oval nuclei with fine chromatin network. Usually 2-5 prominent nucleoli. Cytoplasm deep blue; scant; possesses few or no azurophilic granules. Cytoplasmic vacuolation rare. Progranulocytes nearly always present.</p>

Table 2.--Mean Scores of Cytochemical Reactions of Patients with Acute Leukemias and Compatible Normal Controls

Acute Leukemias	Number of Patients	PAS	Phosphorylase	Acid Phosphatase	Nonspecific Esterase	Lactic Dehydrogenase
ALL	29	134 (62-214)	120 (52-185)	90 (60-131)	71 (38-101)	117 (86-157)
AML	23	88 (48-121)	84 (38-129)	128 (93-155)	144 (120-185)	123 (92-166)
AGL	19	53 (28-102)	46 (28-83)	120 (96-143)	75 (49-88)	121 (107-157)
Normal Controls	Number of Controls	PAS	Phosphorylase	Acid Phosphatase	Nonspecific Esterase	Lactic Dehydrogenase
Lymphocyte	21	39 (26-62)	38 (24-60)	94 (58-120)	68 (42-95)	110 (73-145)
Monocyte	12	74 (47-105)	71 (45-105)	132 (108-150)	140 (105-149)	117 (107-149)
Myeloblast	13	35 (21-67)	33 (20-47)	86 (68-99)	73 (63-87)	91 (72-128)

Abbreviations: ALL=acute lymphocytic leukemia; AML=acute monocytic leukemias; AGL=acute granulocytic leukemia; range of scores in parentheses.

Table 3.--Mean Scores of Alkaline Phosphatase Activity of Mature Neutrophils of Patients with Acute Leukemias and Normal Controls

Acute Leukemias	Number of Patients	Mean Scores of APA
ALL	22	180 (100-272)
AML	15	91 (35-157)
AGL	13	25 (9-44)
Controls	Number of Controls	Mean Scores of APA
Normal	17	82 (60-156)

Abbreviations: ALL=acute lymphocytic leukemia; AML=acute monocytic leukemia; AGL=acute granulocytic leukemia; APA=alkaline phosphatase activity; range of scores in parentheses.

Chart 1.--PAS scores of the primitive cells of patients with acute leukemias.

CHART 1

Chart 2.--PAS scores of the cells of blood and bone marrow of normal controls.

CHART 2

Chart 3.--Acid phosphatase scores of the
primitive cells of patients with acute leukemias.

CHART 3

Chart 4.--Acid phosphatase scores of the cells
of blood and bone marrow of normal controls.

CHART 4

Chart 5.--Alkaline phosphatase scores of the
mature neutrophils of patients with acute leukemias
and of normal controls.

CHART 5

Chart 6.--Nonspecific esterase scores of the primitive cells of patients with acute leukemias.

CHART 6

Chart 7.--Nonspecific esterase scores of the cells
of blood and bone marrow of normal controls.

CHART 7

Chart 8.--Phosphorylase scores of the primitive cells of patients with acute leukemias.

CHART 8

Chart 9.--Phosphorylase scores of the cells of
blood and bone marrow of normal controls.

CHART 9

Chart 10.--Lactic dehydrogenase scores of the
primitive cells of patients with acute leukemias.

CHART 10

Chart 11.--Lactic dehydrogenase scores of the
cells of blood and bone marrow of normal controls.

CHART 11

Legend for Plate 1.

Fig. 1.--Wright's stain showing lymphoblasts
from ALL. X1500

Fig. 2.--Wright's stain showing myeloblasts
from AGL. X1500

Fig. 3.--Wright's stain showing monoblasts from
acute myelomonocytic leukemia. X1500

Fig. 4.--Wright's stain showing monoblasts from
AML. X1500

PLATE I

Legend for Plate II.

Fig. 5.--PAS activity in lymphoblasts from ALL with fine and coarse granules and clumps of reactive material. X1500

Fig. 6.--Similar to Figure 5. PAS activity in lymphoblasts from ALL. X1500

Fig. 7.--PAS reaction in monoblasts from AML showing fine and coarse granules without diffuse staining. X1600

Fig. 8.--PAS activity in a myeloblast from AGL showing diffuse staining. X1500

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PLATE II

Legend for Plate III.

Fig. 9.--PAS activity in a normal lymphocyte from peripheral blood showing moderately coarse granules near the periphery of the cytoplasm. X1500

Fig. 10.--Sudan black reaction in lymphoblasts from ALL showing a few reactive granules. A neutrophil demonstrates intense reactivity. X1600

Fig. 11.--Sudan black reaction in monoblasts from AML showing fine to moderately coarse granules scattered throughout the cells. X1500

Fig. 12.--Sudan black reaction in a myelocyte from normal bone marrow showing intense activity localizing near the nuclear hof. X1500

PLATE III

Legend for Plate IV.

Fig. 13.--Acid phosphatase activity in two lymphoblasts from ALL showing small areas of reactivity near the nuclear hof. X1500

Fig. 14.--Similar to Figure 13. Acid phosphatase activity in lymphoblasts from ALL. X1500

Fig. 15.--Granular localization of acid phosphatase activity in monoblasts from AML. X1500

Fig. 16.--Acid phosphatase activity in a myeloblast from AGL showing reactive granules near the periphery of the cytoplasm. X1500

PLATE IV

Legend for Plate V.

Fig. 17.--Acid phosphatase activity in a normal lymphocyte showing several small areas of reactivity.

Fig. 18.--Strong acid phosphatase activity in a monocyte from normal peripheral blood. X1500

Fig. 19.--Small areas of acid phosphatase activity in a myeloblast from normal bone marrow localizing near the nuclear hof. X1500

Fig. 20.--Nonspecific esterase activity in a lymphoblast from ALL showing reaction granules near the nuclear hof. X1500

PLATE V

Legend for Plate VI.

Fig. 21.--Strong nonspecific esterase activity in monoblasts from AML. X1500

Fig. 22.--Similar to Figure 21. Nonspecific esterase activity in monoblasts from AML. X1500

Fig. 23.--Nonspecific esterase activity in a myeloblast of AGL. Fine and moderately coarse granules are deposited around the periphery of the cytoplasm but are most numerous near the nuclear hof. X1500

Fig. 24.--Nonspecific esterase activity in a normal monocyte showing coarse granules depositing throughout the cell. X1500

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23

PLATE VI

Legend for Plate VII.

Fig. 25.--Nonspecific esterase activity in a normal myeloblast showing a few fine granules scattered throughout the cell but most intense at the nuclear hof. X1600

Fig. 26.--Phosphorylase reaction in lymphoblasts of ALL showing fine and coarse granules and clumps of reactive material. The pattern is similar to the PAS reaction. X1500

Fig. 27.--Phosphorylase activity in monoblasts from AML showing the same coarse activity as was shown by the PAS reaction.

Fig. 28.--Phosphorylase activity in granulocytes from AGL. The myeloblast shows diffuse staining as with PAS. The phosphorylase activity increases with cellular maturation. X1600

PLATE VII

Legend for Plate VIII.

Fig. 29.--Phosphorylase activity in a normal lymphocyte with moderately coarse granules depositing near the periphery of the cytoplasm. The pattern is similar to the PAS activity. X1500

Fig. 30.--Phosphorylase activity in a normal monocyte showing diffuse staining and a few coarse granules. X1500

Fig. 31.--Lactic dehydrogenase activity depositing as fine and coarse granules in lymphoblasts of ALL. X1500

Fig. 32.--Localization of lactic dehydrogenase activity in monoblasts of AML with most intense activity at the nuclear hof. X1500

PLATE VIII

Legend for Plate IX.

Fig. 33.--Intense perinuclear localization of lactic dehydrogenase in a myeloblast of AGL. X1500

Fig. 34.--Localization of lactic dehydrogenase activity as moderately coarse granules in a normal lymphocyte. X1500

Fig. 35.--Lactic dehydrogenase activity in a normal monocyte showing fine and coarse granules in a perinuclear position. X1500

Fig. 36.--Lactic dehydrogenase activity in a normal myeloblast showing a few moderately coarse granules near the periphery of the cytoplasm with most reactivity near the nuclear hof. X1200

PLATE IX

APPENDIX

WRIGHT'S STAIN

The technique used was that of Ackerman.

Reagents

(a) Wright's Stain:

Dissolve a capsule of Wright's dye in 60 ml
of fresh, anhydrous methyl alcohol. Shake.

Add 1 tiny granule of Eosin Y.

Method

1. Place air dried films on a support (e.g., a cork).
2. Cover film completely with stain, and allow it to remain for 60-90 seconds.
3. Dilute stain with distilled water. Add drop by drop, using approximately the same number of drops of water as of the dye. A greenish metallic scum should appear and the margins should show a reddish tint. Allow to remain 3-5 minutes.
4. Wash off with distilled water.
5. Mount in a synthetic mounting medium.

PERIODIC ACID-SCHIFF REACTION

The technique used was that of Ackerman (1960).

Reagents(a) Aqueous Periodic Acid:

Dissolve 500 mg periodic acid crystals in 100 ml distilled water. Store in a dark bottle.

(b) Schiff's Reagent (de Tomasi 1936):

Dissolve 1 g basic fuchsin in 200 ml of boiling distilled water. Cool to exactly 50° C and filter. To the filtrate add 20 ml of 1 N hydrochloric acid. Cool to 25° C. Add 1 g potassium (or sodium) metabisulphite. Keep in the dark for 14-24 hours. Add 2 g activated charcoal. Shake for 1 minute and filter. Store the reagent in the dark at 0-4° C. Allow to reach room temperature before use.

(c) Sulfurous Acid Rinse:

Prepare as follows:

10% aqueous potassium metabisulphite	. .6 ml
1 N hydrochloric acid	.6 ml
Distilled water	110 ml

Method

1. Fix air dried films in formalin vapor for 5 minutes.
2. Rinse briefly in distilled water.
3. Place films in 0.5% aqueous periodic acid for 10 minutes.
4. Wash in distilled water.
5. Immerse in Schiff's reagent for 20 minutes.
6. Wash in 3 changes of sulfurous acid rinse, 2 minutes each.
7. Wash in running tap water 5-10 minutes.
8. Counterstain with 0.5-1% aqueous methyl green for 2 minutes. The methyl green must be purified by repeated chloroform extraction.
9. Wash in running water.
10. Blot dry and mount with synthetic mounting medium.

Results

Intense red purple reaction indicates glycogen; deep pink the presence of glycoprotein or mucopolysaccharides.

SUDAN BLACK B REACTION

The technique used was that of Sheehan and Storey (1947).

Reagents

(a) Sudan Black B Solution:

Dissolve 0.3 g of Sudan black B powder in 100 ml absolute ethanol.

(b) Buffer Solution:

Dissolve 16 g crystalline phenol in 30 ml absolute ethanol. Add this to 100 ml distilled water in which 0.3 g hydrated disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) has been dissolved.

(c) Working Stain Solution:

Add 40 ml buffer solution to 60 ml Sudan black B solution. Filter by suction. Store in cold.

The stain, so prepared, can be used for 2-3 months.

Method

1. Fix air dried films in formalin vapor for 5-10 minutes.
2. Wash in running tap water for 10 minutes.
3. Immerse in working stain at room temperature for 1 hour.

4. Wash with 70% ethanol for 2-3 minutes to remove excess dye.
5. Wash with tap water for 2 minutes.
6. Blot dry and counterstain with Leishman's stain.
7. Rinse in distilled water.
8. Mount in synthetic mounting medium.

Results

Sudanophilia can appear as faint localized reactivity to strong localized or heavy overall activity.

ACID PHOSPHATASE REACTION

The technique used was that of Goldberg and Barka (1962).

Reagents(a) Formol-calcium:

Dissolve 1 g anhydrous calcium chloride in 60 ml distilled water. Add 10 ml formaldehyde and adjust to pH 7.0-7.2 with 1 N sodium hydroxide. Add distilled water to make 100 ml. Check pH of the fixative weekly and readjust if necessary. Store in refrigerator.

(b) Pararosanilin Solution:

Dissolve 1 g pararosanilin hydrochloride in 20 ml distilled water and 5 ml concentrated hydrochloric acid with gentle warming. Cool and filter. May be stored at room temperature.

(c) Sodium Nitrite Solution:

Dissolve 0.4 g sodium nitrite in 10 ml of distilled water. Prepare fresh each day.

(d) Michaelis Veronal Acetate Buffer Stock

Solution (0.15 M):

Mix 9.714 g sodium acetate $\cdot 3H_2O$ (5.85 g of anhydrous salt) and 14.714 g sodium barbital in carbon dioxide-free distilled water to a final volume of 500 ml.

(e) Stock Substrate Solution:

Dissolve 10 mg naphthol AS-BI phosphate in 1 ml N, N-dimethylformamide (DMF). If larger volumes for stock solutions are needed store in cold.

(f) Naphthol AS-BI Phosphate Hexazonium Pararosanilin Solution:

Mix 2.5 ml Michaelis veronal acetate stock buffer solution with 0.5 ml naphthol AS-BI phosphate stock solution and 6.0 ml distilled water. Mix 0.4 ml pararosanilin solution with 0.4 ml sodium nitrite solution in a test tube. Add the pararosanilin-sodium nitrite solution to the substrate buffer mixer. Adjust this solution to pH 5 with 1N sodium hydroxide. This solution should be freshly made prior to use.

Method

1. Air dry blood films from 10-30 minutes at room temperature.
2. Fix in formol vapor for 1 minute followed by formol-calcium for 3 minutes.
3. Rinse thoroughly in distilled water.
4. Place in freshly made naphthol AS-BI phosphate hexazonium pararosanilin solution for 2-3 hours at room temperature or 1-1 1/2 hours at 37° C.
5. Rinse thoroughly in distilled water.
6. Counterstain if desired with 1% methyl green in Michaelis veronal acetate buffer, pH 4 for 30-60 seconds.
7. Rinse in distilled water. Blot dry and mount with synthetic mounting medium.

Results

Sites of acid phosphatase activity appear red.

ALKALINE PHOSPHATASE REACTION

The technique used was that of Ackerman (1962).

Reagents

(a) Dimethylformamide (DMF):

Purify the DMF by shaking with activated charcoal. Filter through Linde Molecular Sieve, type 4A, into a dry bottle containing more molecular sieve. Store in refrigerator.

(b) Substrate Stock Solution:

Dissolve 12.5 mg of naphthol AS-MX phosphate in 5 ml of purified dimethylformamide. Add 20 ml distilled water and enough 1 M sodium carbonate solution to adjust the substrate solution to pH 8. Add 130 ml distilled water and 95 ml of 0.2 M Tris buffer pH 8.3. The substrate solution should stand for at least 24 hours before use. It must be refrigerated and thoroughly mixed before use.

(c) Incubation Solution:

Prior to use add 1 mg Fast blue RR salt to each ml of substrate stock solution and filter.

Magnesium or manganese ions may be added for activation.

(d) Formol-methanol:

Mix 10 ml of formaldehyde in 90 ml methanol.

Method

1. Fix films in cold formol-methanol for 30 seconds.
2. Wash in running tap water for 10 seconds.
3. Place in the incubating solution for 45 minutes at room temperature.
4. Wash in distilled water.
5. Counterstain with 1% aqueous neutral red for 30 seconds.
6. Wash in running distilled water.
7. Blot dry and mount in Kaiser's glycerine-jelly.

Results

Sites of alkaline phosphatase appear blue. Mature neutrophils, reticular, and reticulo-endothelial cells are only reactive cells in normal blood and bone marrow.

NONSPECIFIC ESTERASE REACTION

The technique used was that of Wachstein and Wolf (1958).

Reagents

(a) Substrate Solution:

1% propylene glycol in 0.2 M

Phosphate buffer (pH 6.9). 40 ml

1% naphthol AS acetate 0.4 ml

Fast blue BB salt. 80 mg

The substrate must be freshly prepared prior
to use.

Method

1. Fix air dried films in formalin vapor for
15 minutes.
2. Wash in distilled water.
3. Immerse in freshly prepared substrate at room
temperature for 90 minutes.
4. Rinse in distilled water.
5. Counterstain for 10 minutes with 0.1% nuclear
fast red in 5% aluminum sulphate solution.
6. Rinse in distilled water.
7. Mount in Kaiser's glycerine-jelly.

Results

Sites of esterase activity appear blue with the deposition of dust-like and larger granular dye deposits in the cytoplasm.

PHOSPHORYLASE REACTION

The technique used was slightly modified from that of Takeuchi and Kinoshita (1956) and Eränkö and Palkama (1961).

Reagents

(a) Substrate Solution:

Glucose-1-phosphate	150 mg
Adenosine-5-phosphate	10 mg
Glycogen	10 mg
Sodium fluoride	180 mg
Polyvinyl pyrrolidone	900 mg
Insulin 40 i.u./ml	0.1 ml
Ethanol	2 ml
Acetate buffer 0.1 M pH 5.9 . . .	10 ml

The solution must be freshly made and filtered.

(b) Gram's Iodine Solution:

Iodine	1 g
Potassium iodide	2 g
Sucrose solution 0.32 M	300 ml

(c) Iodine-glycerine Mounting Medium:

Mix 1 part iodine solution with 5 parts of glycerine.

Method

1. Allow air dried films to fix for 30 seconds in 70% acetone. Blot dry.
2. Incubate in the substrate solution for 2-3 hours at 37° C.
3. Immerse in 40% ethanol for 2 minutes.
4. Allow to air dry.
5. Place in 0.32 M sucrose for 5 minutes.
6. Place in Gram's iodine solution for 8 minutes.
7. Mount in iodine-glycerine.

Results

Phosphorylase activity appears dark-blue, violet-blue in color. The reaction closely parallels the PAS reaction for glycogen and glycoprotein in both normal and abnormal blood and bone marrow cells.

LACTIC DEHYDROGENASE REACTION

The technique used was modified from those described by Ackerman (1960), Quaglino and Hayhoe (1960), and Balogh and Cohen (1961). Polyvinyl pyrrolidone was used in the substrate as recommended by Scarpelli and Pearse (1958).

Reagents

(a) Sodium Azide Stock Solution:

Dissolve 0.065 g sodium azide crystals
(M.W. 65.02) in 10 ml distilled water. Makes a
0.1 M stock solution.

(b) Sodium Lactate Stock Solution:

Dissolve 1.12 g sodium lactate crystals
(M.W. 112.06) in 10 ml distilled water. Makes a
1 M stock solution.

(c) Substrate Solution:

Phosphate buffer 0.2 M, pH 7.2	. . . 10 ml
Sodium lactate 0.2 M	 10 ml
D.P.N.	 10 mg
Sodium azide 0.1 M	 1.5 ml
P.V.P.	 700 mg

Add 10 mg Nitro B.T. dissolved in 10 ml distilled water. Substrate solution may be kept in the freezer and thawed prior to use.

Method

1. Fix air dried films with 60% acetone for 30-60 seconds.
2. Blot dry.
3. Incubate in substrate solution for 1-2 hours at 37° C.
4. Rinse in distilled water.
5. Counterstain with 1% aqueous neutral red for 30 seconds.
6. Blot dry and mount in Kaiser's glycerine-jelly.

Results

Sites of lactic dehydrogenase activity appear blue-purple.

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