

THE CLINICAL APPLICATION OF A MODIFIED AZO-DYE TECHNIC FOR THE DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY IN NEUTROPHILS

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THE phosphatases are a group of enzymes that release orthophosphoric acid from many phosphate esters and, to a lesser extent, participate in the reverse reaction. These enzymes differ in their optimal pH and, on this basis, they may be classified as either acid or alkaline phosphatases. In 1939, Gomori¹ and Takamatsu² independently described a method for the cytochemical demonstration of alkaline phosphatase. Since that time the distribution of alkaline phosphatase in the cells of the hematopoietic system has been the subject of much study. In 1944, Menten, Junge, and Green³ described an azo-dye method for the demonstration of the enzyme. The basic reactions in the technic are the release of naphthol from an alpha-naphthyl phosphate substrate, and its combination with a diazotized amine to form an azo dye. This dye forms a colored precipitate at the site of action of the alkaline phosphatase within the cells.

Reports⁴⁻⁸ differ both as to the types of cell that contain alkaline phosphatase and as to the distribution of the enzyme within the individual cells. Nevertheless, most authors agree that it is the alteration in enzyme content of the segmented neutrophils which offers the most useful diagnostic information. In chronic granulocytic leukemia the enzyme activity of the segmented neutrophils has been shown⁴⁻¹⁴ to be greatly reduced or absent, whereas in other forms of granulocytosis this activity is frequently increased. Polycythemia vera,¹⁵ myelofibrosis,^{13,14} Hodgkin's disease¹⁵ and pregnancy¹⁶ are also associated with increased neutrophil alkaline phosphatase.

The purpose of this paper is to present a modified azo-dye technic for the cytochemical measurement of alkaline phosphatase, and to discuss its use as a diagnostic aid.

Method

A modification of the azo-dye methods described by Kaplow⁷ and by Hayhoe and Quaglino⁸ was used for the semiquantitative measurement of alkaline phosphatase in segmented neutrophils.

Films of freshly drawn blood are spread on chemically clean glass slides; the enzyme activity of the neutrophils falls rapidly if the blood is drawn into an anticoagulant solution. The films are fixed for 90 seconds in a mixture of 10 per cent formalin in absolute alcohol at 5 C. A fixation time of 90 seconds was found to lead to a more distinct staining reaction than a fixation time of 30 seconds.

Fixed blood films can be stored overnight prior to staining, however unfixed blood films are unsatisfactory if stored for this length of time. The fixative must be stored at -20 C. and should be freshly prepared each week. After fixation, the blood films are rinsed in tap water and stained for 10 minutes at room temperature with the following mixture:

Sodium alpha-naphthyl phosphate.....	35 mg.
Brentamine Fast Garnet*.....	35 mg.
0.05M propanediol buffer.....	35 ml.

This mixture must be prepared immediately before use and should be filtered directly onto the slide. After rinsing in distilled water the blood film is counterstained with Mayer's hematoxylin for 10 minutes. The estimation of alkaline phosphatase activity was found to be simpler when Mayer's hematoxylin rather than methyl green was used as a nuclear counterstain. The stained film is mounted in Kaiser's glycerine jelly, a mounting medium that considerably slows the rate at which the azo dye fades. The color can be preserved for even longer periods if the stained film is fixed in absolute alcohol prior to mounting.

When alkaline phosphatase is present in a cell (*Fig. 1*) it is represented by a red-brown precipitate in the cytoplasm. The amount of this precipitate varies

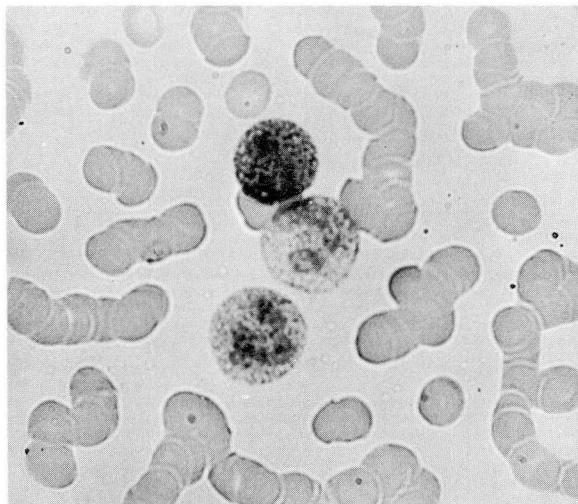


Fig. 1. A photomicrograph of three segmented neutrophils showing alkaline phosphatase activity represented by a (red-brown) precipitate of varying density in the cytoplasm. The upper neutrophil is graded 4+, the central neutrophil, 2+, and the lower neutrophil, 3+. Magnification x 1000.

from a faint diffuse brown coloration to a dense aggregation of red-brown granules. Using the method of scoring described by Kaplow,⁷ this variation in staining can be used roughly to quantitate the amount of enzyme that is present in the cell. One hundred consecutive segmented neutrophils are individually scored in a

*Imperial Chemical Industries Ltd., England.

range from 0 to 4+. These individual scores are totaled and the resulting figure is the score for the blood being studied. Thus the range of possible scores is 0 to 400.

Results

The various disorders and the number of patients studied are listed in *Table 1*. The individual alkaline phosphatase scores obtained in the larger groups are charted in *Figure 2*.

Table 1.—*The number of patients* studied and their respective diagnoses*

Diagnosis	Patients studied, number
Normal	30
Chronic granulocytic leukemia	13
Other chronic leukemias	6
Acute leukemias	7
Benign leukocytosis	15
Polycythemia vera	30
Anoxemic erythrocytosis	10
Myelofibrosis and allied disorders	6
Hodgkin's disease	20
Other lymphomas	8
Disseminated lupus erythematosus	10
Primary refractory anemia	4
Pernicious anemia in relapse	2
Hyperthyroidism	3
Hypothyroidism	3
Pregnancy	2
Granulocytopenia (drug-induced), acquired hemolytic anemia, thrombotic thrombopenic purpura, sickle-cell anemia, infectious mononucleosis	1 each

*Many of these patients were under the care of Dr. John D. Battle, Jr., and Dr. James S. Hewlett, of the Department of Hematology.

Alkaline phosphatase activity was seen in neutrophilic myelocytes, metamyelocytes, band forms, and, more commonly, in segmented neutrophils. The only other cells to show activity were the reticuloendothelial cells of the bone marrow. Alkaline phosphatase was found to be restricted to the cytoplasm and was never seen in the nucleus. Since the counterstained nucleus was visible even in cells

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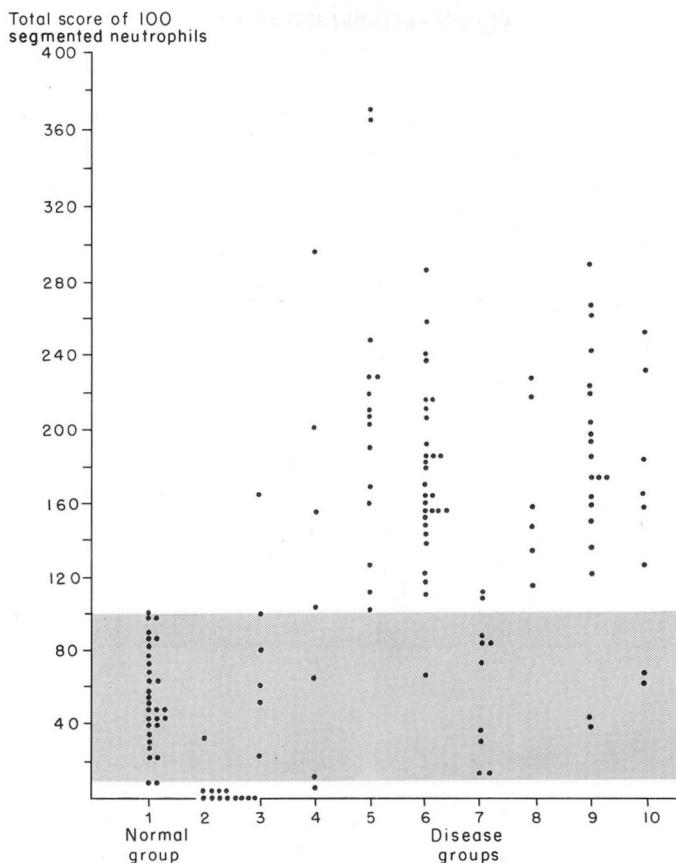


Fig. 2. A chart showing the individual alkaline phosphatase scores obtained in a group of normal persons and in patients with various diseases. The normal range is shaded. Key: 1 = normal; 2 = chronic granulocytic leukemia; 3 = other chronic leukemias; 4 = acute leukemias; 5 = non-leukemic leukocytosis; 6 = polycythemia vera; 7 = anoxemic erythrocytosis; 8 = other myeloproliferative disorders; 9 = Hodgkin's disease; 10 = other lymphomas.

graded 4+, the identity of the individual cells was seldom in doubt. The only exception was the basophil, which was not clearly recognizable. Eosinophils, which were recognized by their nuclear structure, showed no alkaline phosphatase activity.

Normal persons. Peripheral blood films from 30 normal persons were studied, and the alkaline phosphatase scores ranged from 8 to 100. No cells with a 4+ rating were seen in this group, and less than 1 per cent of the neutrophils was rated 3+. The greatest number of cells rated 3+ in any one normal blood film

was 3 per cent. This was in distinct contrast to that found in diseases associated with a high score (Fig. 3).

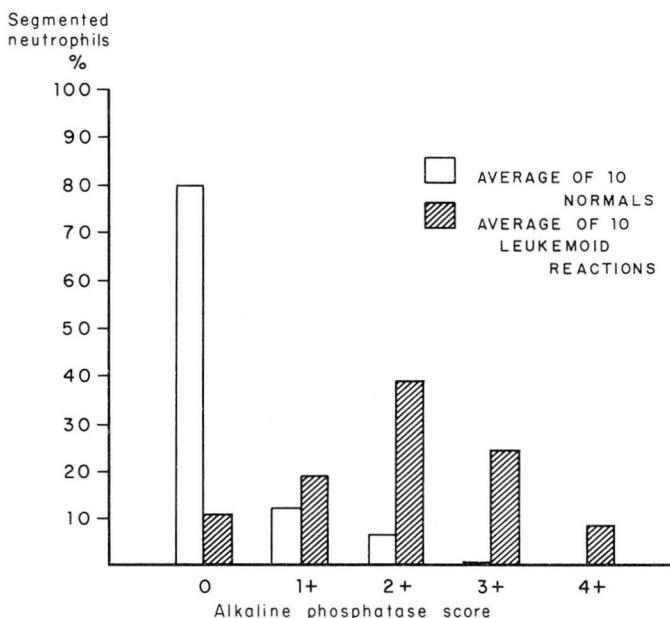


Fig. 3. A comparison of the distribution of neutrophil alkaline phosphatase scores (0 to 4+) in a group of 10 normal persons and a group of 10 patients with leukemoid reactions.

During the study, pharyngitis developed in two of the normal subjects and, although the leukocyte counts remained within normal limits, the alkaline phosphatase scores rose to 130 and 190. When the symptoms subsided the scores returned to 83 and 36, respectively.

Chronic granulocytic leukemia. Eight of thirteen patients with chronic granulocytic leukemia had scores of zero, and 12 had scores of 3 or less. Four of the 12 patients, untreated at the time of study, had leukocyte counts ranging from 89,500 to 373,000 per cubic millimeter. The remaining eight patients had been under treatment for from four months to four years and had leukocyte counts ranging from 4,800 to 18,900 per cubic millimeter. The one patient with a normal score of 33 had been treated with busulfan for six months and had a normal leukocyte count.

Other leukemias. The five patients with chronic lymphocytic leukemia showed a wide range of scores from 22 to 163. There was no relationship between the alkaline phosphatase score and the total leukocyte count, the neutrophil count, or the stage of the disease. One patient with chronic monocytic leukemia had a score of 60.

Seven patients with acute leukemia also showed a wide range of scores. Two patients with acute granulocytic leukemia had scores of 6 and 154; four patients with acute lymphocytic leukemia had scores of 11, 64, 102, and 201; one patient with acute monocytic leukemia had a score of 295.

Nonleukemic leukocytosis. Nonleukemic leukocytosis almost invariably was associated with alkaline phosphatase scores above the normal range. Nine patients with leukocytosis due to infection had scores ranging from 111 to 372. In this group there was a rough correlation between the neutrophil count and the alkaline phosphatase score. Three patients with carcinomatosis and leukocytosis had scores of 169, 218, and 367. Two other patients with carcinomatosis and normal leukocyte counts had scores of 140 and 216; these two scores are not included in Figure 2. All five patients with carcinomatosis showed some evidence of infection.

Three patients with leukocyte counts of 16,000, 20,000, and 31,000 per cubic millimeter showed no evidence of any underlying disease and had high leukocyte counts for at least one year. Their scores were 160, 249, and 103, respectively.

Polycythemia vera and anoxemic erythrocytosis. Thirty cases of polycythemia vera were studied and with one exception (score of 67) the scores were more than 110. Twenty of these patients had leukocyte counts of less than 10,000 per cubic millimeter, and their scores ranged from 67 to 258 with an average of 170. Ten patients had leukocyte counts of more than 10,000 per cubic millimeter, and their scores ranged from 143 to 288 with an average of 187. These differences are of little significance. Similarly, a comparison of the scores obtained in treated and in untreated patients showed no significant difference. In most instances the treated patients had received radioactive phosphorus therapy for months or years, and had normal peripheral blood counts. The score of 67 was obtained in a patient who has required no treatment for four years.

In contrast to the findings in patients with polycythemia vera, 10 patients with anoxemic erythrocytosis had scores ranging from 11 to 112 with an average of 65.

Other myeloproliferative disorders. The group of six patients with myeloproliferative disorders, other than polycythemia vera or chronic granulocytic leukemia, comprised: three patients with myelofibrosis and myeloid metaplasia; one patient with myelofibrosis and myeloid metaplasia secondary to a metastasizing carcinoma of the breast; one patient with myelofibrosis, myeloid metaplasia, and mild polycythemia vera; and one patient with megakaryocytic myelosis. All six patients had alkaline phosphatase scores above the normal range, the lowest being 117 and the highest 229.

Hodgkin's disease and other lymphomas. Of 20 patients with Hodgkin's disease, 18 showed an increased alkaline phosphatase activity. There was no correlation between the alkaline phosphatase score and the total leukocyte count. Thus the six patients who had total leukocyte counts of less than 3,000 per cubic millimeter had scores ranging from 124 to 291. No correlation was found between the

enzyme activity, the stage of the disease, or the response to treatment.

Seven patients with lymphosarcoma were studied and their scores ranged from 65 to 254, five being above the normal range. One patient with a follicular lymphoma had a score of 196.

Other diseases. Ten patients with disseminated lupus erythematosus had alkaline phosphatase scores ranging from 61 to 258. Many of these patients had one or more complications such as hemolytic anemia, circulating anticoagulant, or thrombocytopenia. Two patients with untreated pernicious anemia had low scores of 8 and 12; their total leukocyte counts were 3,900 and 4,400 per cubic millimeter. Four patients with primary refractory anemia had scores of 5, 16, 24, and 111. Three patients with hyperthyroidism had scores of 33, 44, and 224. Three patients with myxedema had scores of 29, 49, and 220. Two women in the second and third trimesters of pregnancy had scores of 271 and 288. Single instances of acquired hemolytic anemia, sickle-cell anemia, thrombotic thrombopenic purpura, and infectious mononucleosis had scores within the normal range. One patient with rheumatoid arthritis, in whom a drug-induced granulocytopenia developed, was studied on two occasions. When initially studied the total leukocyte count was 2,100 per cubic millimeter with 10 per cent neutrophils. At that time the alkaline phosphatase score was 224. Two weeks later, when the total leukocyte count had risen to 8,000 per cubic millimeter with 54 per cent neutrophils, the alkaline phosphatase score was 93.

Discussion

The semiquantitative estimation of the alkaline phosphatase content of mature neutrophils by histochemical means has become established as a useful tool in the differential diagnosis of various hematologic disorders and certain other diseases. At present, the most useful application of this test appears to be in the differential diagnosis of chronic granulocytic leukemia and leukemoid reactions. The absence or great reduction of alkaline phosphatase activity is characteristic of chronic granulocytic leukemia, while the scores obtained in leukemoid reactions are normal or more commonly elevated. Although Tanaka, Valentine, and Fredricks¹⁷ have shown that diseases other than chronic granulocytic leukemia may be associated with low enzyme activity, we have not found scores of 3 or less in any disease other than chronic granulocytic leukemia. The scores in the lower limits of the normal range occurred either in normal persons or in patients having diseases that are unlikely to be confused with chronic granulocytic leukemia. With one possible exception we have observed no return of alkaline phosphatase activity during a remission of chronic granulocytic leukemia. However, a return of enzyme activity has been reported by some authors.^{8,13}

Myeloproliferative disorders, other than chronic granulocytic leukemia, such as myelofibrosis with myeloid metaplasia, megakaryocytic myelosis, and polycythemia vera are associated with high alkaline phosphatase scores. The high scores found in polycythemia vera often help to distinguish this form of erythro-

cytosis from the anoxemic type. A return to normal values following the successful therapy of polycythemia vera has been reported.¹² However, even in those patients who have been under excellent control for several years we have found only one normal score.

In contrast to the absent or reduced alkaline phosphatase activity found in chronic granulocytic leukemia, neutrophil leukocytosis due to any other cause is almost invariably associated with increased activity. Even a mild infection, unassociated with a significant leukocytosis, such as occurred in two of the group of normal persons, may be associated with a high score. The occurrence of increased neutrophil alkaline phosphatase under many nonspecific circumstances thus makes the interpretation of scores above the normal range difficult.

The increased alkaline phosphatase activity found in the various types of lymphoma may be of some diagnostic value. Hayhoe and Quaglino⁸ found high scores in patients with Hodgkin's disease, and normal scores in patients with other types of lymphoma. We have been unable to make this distinction for, although we obtained consistently high scores in patients with Hodgkin's disease, we also obtained high scores in five of seven patients with other types of lymphoma.

No other useful diagnostic application for this test was found among the other conditions we have studied, with the possible exception of pregnancy. Quigley, Dawson, Bong, and Custer¹⁶ have recently reported the interesting observation of high alkaline phosphatase scores at all stages of pregnancy, and they discuss the possibility of applying this finding to the diagnosis of early pregnancy. The only two pregnant women we have studied were in the second and third trimesters of pregnancy and had scores of 271 and 288.

Various theories have been propounded to explain the changes in the alkaline phosphatase content of the neutrophils that occur in various disorders. The different opinions that exist concerning the types of cell that contains alkaline phosphatase are no doubt in part due to differences in technic. These technics may not be measuring identical members of the alkaline phosphatase group of enzymes.¹⁸ Trubowitz, Feldman, Benante, and Hunt¹⁹ have reported high alkaline phosphatase scores following the administration of nitrogen mustard. They suggest that the high scores resulted from a cessation of production of young neutrophils, and the persistence in the peripheral blood of older neutrophils with high alkaline phosphatase activity. The same authors²⁰ noted higher scores in the segmented neutrophils of the peripheral blood than of the bone marrow. Both these findings indicate that alkaline phosphatase activity increases with the age of the neutrophil. Our findings in the patient with the transient drug-induced granulocytopenia may have a similar explanation. It can be postulated that the high score reflected an older population of neutrophils surviving from the period prior to bone marrow depression, and with the return of bone marrow activity and the production of younger neutrophils, the score returned to the normal range.

The leukocytosis that occurs in response to infection may, in part, result from

the release of relatively old neutrophils from sites of sequestration. However, the majority are younger cells arising directly from the bone marrow, and would not be expected to contain an increased amount of alkaline phosphatase if their age were the only factor. The high score that accompanies a leukocytosis due to infection must, therefore have some other explanation. Valentine and his associates²¹ showed a direct correlation between adrenocortical activity and neutrophil alkaline phosphatase content. They suggest that this is a common denominator in various disorders, including pyogenic infections. The administration of 17-hydroxycorticosteroids will also increase the phosphatase activity of the neutrophils. We have studied a patient who received 200 gm. of prednisolone daily, and after three days of such treatment the enzyme score had risen from 79 to 143. Apart from its adrenocortical stimulating effect, a pyogenic infection may also increase the metabolic rate, and this might be reflected at the cellular level by an increase in alkaline phosphatase activity. To test this hypothesis, a small group of hyperthyroid and hypothyroid patients were studied. However, there was no significant correlation between metabolic rate and alkaline phosphatase activity of the neutrophils.

It is evident that more precise interpretation of the changes that occur in the alkaline phosphatase content of the neutrophils must await further knowledge concerning the physiologic factors affecting the enzyme at the cellular level. Nevertheless, useful clinical information can be gained from a study of the alkaline phosphatase content of the neutrophils.

Summary

A modified azo-dye technic for the cytochemical demonstration of the enzyme, alkaline phosphatase, in neutrophils is described.

This technic has been applied on a semiquantitative basis to peripheral blood films taken from normal persons and from patients with various disorders.

The absent or reduced alkaline phosphatase activity of the segmented neutrophils in chronic granulocytic leukemia, serves to distinguish this disease from other myeloproliferative disorders and from leukemoid reactions. Polycythemia vera with its increased alkaline phosphatase activity can be distinguished from other forms of erythrocytosis. The lymphomas are also associated with increased neutrophil alkaline phosphatase activity.

The findings in various other disorders are discussed with particular reference to the pitfalls that exist in interpreting increased alkaline phosphatase activity.

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