Histochemical and Biochemical Studies on Leukocyte Alkaline Phosphatase Activity

By E. Wiltshaw and W. C. Moloney

The development of histochemical methods in the past ten years has resulted in a more fundamental and dynamic approach to many cytologic problems, particularly in the field of hematology. More recently, efficient methods for separating leukocytes from the peripheral blood have been available, permitting biochemical measurements of enzyme and other cellular constituents. The use of both histochemical and biochemical methods have demonstrated marked differences in enzyme activity in cells that appear morphologically similar. Of even greater importance, these procedures may be employed in the evaluation of physiologic and pathologic influences on certain leukocytic activities and functions. Initial investigations into some aspects of these problems are presented in this report.

METHODS

Histochemical: Gomori's method for alkaline phosphatase, with modifications, was used throughout. Smears or imprints were taken on cover slips or slides, air-dried, and then fixed in 95 per cent ethyl alcohol. Celloidin was not used on the preparations since it interfered with the penetration of the counterstain. The specimens were then incubated for two hours at 37 C. in a barbital buffered medium containing Beta-glycerophosphate, calcium chloride and magnesium sulphate at a pH of 9.2. Next the cover slips were thoroughly washed in dilute calcium chloride solution and immersed in 1 per cent cobalt nitrate for five minutes. Trials with various dilutions of the cobalt solution from 0.1 per cent to 2 per cent were made and while good results were obtained with as little as 0.1 per cent, 0.5-1 per cent solutions were found to be optimal for consistent results rather than the 2 per cent suggested by Gomori. This alteration reduced artifacts and diffusion difficulties as well as excessive cobalt precipitations on the specimens. Following cobalt immersion, the smears were washed in tap water and placed in dilute ammonium sulphide for a further five minutes after which they were washed once more, dried, counterstained with Wright or Wright-Giemsa and mounted. This technic gave good cytologic and histochemical visualization. As shown in Plate 1 (fig. 2), the black precipitate of cobalt sulphide was deposited in the cytoplasm at the site of alkaline phosphatase activity. The intensity of the reaction could be roughly quantitated on a plus/minus to 4 plus basis.

Biochemical: The method for determining alkaline phosphatase on separated white blood cells was that used by Valentine and Beck2 without modification. However the method of cell separation was that suggested by E. Klein.3 This technic involved the placing of 0.3 cc. of EDTA (sequestrene) and 0.5 cc. of especially selected dextran into the barrel of a syringe for every 10 cc. of whole blood withdrawn. The syringe was then inverted at least 5 times to insure adequate mixing and then clamped nozzle uppermost to allow for separation. After standing for 30 minutes, the plasma containing white cells and platelets was forced out of the syringe into a test tube. This method provided a rich white blood cell suspension usually containing less than 1 per cent red cells. After washing the cells in saline

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and centrifuging twice, only minimal platelet contamination was found in the last suspension, an important factor in obtaining leukocytes free from agglutination.

### Materials

As shown in table 1, 150 patients were studied and divided into various disease groups. The materials consisted of peripheral blood and bone marrow smears and imprints or smears from spleens and lymph nodes. In all, 350 histochemical procedures were carried out on these specimens and in many instances biochemical determinations were made on separated leukocytes of these patients. The major part of this investigation was concerned with leukemia and allied disorders; however, in addition normal controls and various other dis-
orders were studied. Included in this miscellaneous group were hemolytic, hypochromic and aplastic anemias, purpuras and lupus erythematosus. Although some interesting observations were made, no specific features of alkaline phosphatase activity were found in this heterogeneous group. While 7 cases of megaloblastic anemia were investigated, intercurrent infections or other complicating factors made it impossible to interpret the findings.

**General Observations**

Wachstein and others\(^4, 5\) state that in the peripheral blood only the segmented
and band form neutrophils contain alkaline phosphatase. An adequate counterstain and a short incubation period made it apparent indeed that this enzyme is demonstrable only in the cytoplasm of these peripheral blood cells and that it does not appear in the nucleus. It has also been said that histochemical methods for alkaline phosphatase were unsatisfactory in bone marrow preparations, but this has not been our experience, if thin marrow smears are used. Except for an occasional metamyelocyte and for a rare “histiocytic-like” cell (see Plate 2) found in certain disease states, the alkaline phosphatase was confined to the adult and band neutrophil in the bone marrow. This was true also in the spleen.
and lymph node material as well as in the cells obtained from various body fluids.

Normal Peripheral Blood

In normal subjects the majority of polymorphonuclear leukocytes showed no stain for alkaline phosphatase. Approximately 20–40 per cent of cells revealed a plus minus to 2 plus staining intensity. Biochemical determinations gave an
average result of 21.9 mg. of P liberated per hour by 10^6 leukocytes, with a range of 18–26 mg.

**Infection**

In all cases of pyogenic infection the leukocytes showed greatly increased alkaline phosphatase activity. In one case of Weil's disease and in several cases of pulmonary tuberculosis, marked elevation of enzyme activity was also ob-

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**PLATE 1: FIG. 2.**—Peripheral blood smear from a case of myeloid metaplasia stained for alkaline phosphatase by the Gomori technic.
LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY

TABLE 2

Showing the distribution and total content of Alkaline Phosphatase in cases of multiple myeloma and chronic lymphatic leukemia compared with that in normals and chronic myelogenous leukemic cases.

<table>
<thead>
<tr>
<th>DIAGNOSIS</th>
<th>NO CASES</th>
<th>AV NO POLYS PER CMM</th>
<th>AV% NEG CELLS</th>
<th>AV% POS CELLS</th>
<th>AVERAGE DISTRIBUTION</th>
<th>LEUKOCYTE ALK PHOS*</th>
<th>POLY MORPH ALK PHOS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td>8</td>
<td>4,805</td>
<td>640</td>
<td>3560</td>
<td>4.0</td>
<td>80</td>
<td>5.6</td>
</tr>
<tr>
<td>CHRONIC LYMPHATIC LEUKEMIA</td>
<td>3</td>
<td>4,241</td>
<td>3510</td>
<td>6490</td>
<td>2.0</td>
<td>283</td>
<td>40</td>
</tr>
<tr>
<td>MULTIPLE MYELOMA</td>
<td>8</td>
<td>4,531</td>
<td>2835</td>
<td>7165</td>
<td>1.4</td>
<td>234</td>
<td>6.7</td>
</tr>
<tr>
<td>CHRONIC MYELOGENOUS LEUKEMIA</td>
<td>3</td>
<td>57,307</td>
<td>9870</td>
<td>130</td>
<td>1.0</td>
<td>0.3</td>
<td>7.90</td>
</tr>
</tbody>
</table>

*Alkaline Phosphatase activity expressed as mgs of P liberated per hour by 10⁶ leukocytes

It was interesting that in 3 cases of infectious mononucleosis, the alkaline phosphatase activity was negligible in the neutrophils of the peripheral blood.

Leukemoid Reactions

Neutrophil leukemoid reactions are seen in a variety of clinical disorders other than in response to pyogenic infection. In this series 4 cases of myeloid metaplasia, 6 cases of carcinoma and 12 cases of cirrhosis of the liver were studied (table 1). Other observers have shown that the polymorphonuclear leukocytes under these conditions have high cellular alkaline phosphatase activity by histochemical and biochemical measurements. Since these disorders may simulate chronic myelogenous leukemia closely these determinations are of practical value as an aid in differential diagnosis.

Leukemia

It is well known that in acute leukemia the immature neutrophilic leukocytes are devoid of alkaline phosphatase. In chronic myelogenous leukemia this enzyme cannot be demonstrated except as a faint plus/minus stain in an occasional cell. Biochemical readings were also very low with an average of 7.8 mg. of P liberated per hour by 10⁶ white blood cells.

Charts 1 and 2 and Plate 1 (figs. 1 and 2) demonstrate clearly that leukocytes that are morphologically similar may contain markedly dissimilar alkaline phosphatase activity. The high content of this enzyme in myeloid metaplasia and pyogenic infection is in marked contrast to the low normal cell value and the almost negligible alkaline phosphatase activity found in the leukocytes of chronic myelogenous leukemia.

The biochemical measurement alone may be misleading as an index of unit neutrophil alkaline phosphatase activity. This became apparent in the 9 multiple myeloma and 10 chronic lymphatic leukemia cases studied in this series. While alkaline phosphatase activity appeared normal by the biochemical technic, the histochemical method showed intense staining of almost every adult neutrophil. By correlating the biochemical readings to 10⁶ segmented and band
form neutrophils it became obvious that in these diseases unit cell alkaline phosphatase activity was greatly elevated (table 2).

Valentine⁸ has shown by biochemical methods that the high leukocyte alkaline phosphatase activity in pyogenic infections diminishes as the clinical condition improves. These findings were confirmed in our studies by both biochemical and histochemical methods and are shown graphically in Chart 3.
Experimental Results

The function of alkaline phosphatase in the neutrophil is unknown. It has been postulated by Valentine and others that this enzyme may play a role in the reaction to infection or other stress phenomena. Another possibility is that this enzyme takes part in the intrinsic metabolic activity of the cell, such as glycogenesis or nucleic acid synthesis. It has occurred to us that the neutrophil may be a vehicle for the transport of this enzyme from the bone marrow to other sites. Therefore, attempts were made to study the relative concentration of alkaline phosphatase in the leukocytes of peripheral blood and bone marrow when they were drawn simultaneously. Although errors such as dilution of bone marrow aspirates cannot be excluded, it appears, so far, that histochemically and biochemically there is no consistent difference between these materials (see table 3). However, further studies are being undertaken to test this hypothesis.
TABLE 3 A comparison of Alkaline Phosphatase activity of blood and bone marrow in various conditions

<table>
<thead>
<tr>
<th>DIAGNOSIS</th>
<th>CASE NO</th>
<th>SOURCE</th>
<th>AVERAGE DISTRIBUTION</th>
<th>% POSITIVE CELLS</th>
<th>% NEGATIVE CELLS</th>
<th>LEUKOCYTE ALK PHOS</th>
<th>POLYMORPH ALK PHOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td>1</td>
<td>PB</td>
<td>8 11 16 1</td>
<td>36</td>
<td>64</td>
<td>34.8</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM</td>
<td>14 30 21 1</td>
<td>66</td>
<td>34</td>
<td>34.1</td>
<td>81</td>
</tr>
<tr>
<td>MULTIPLE MYELOMA</td>
<td>2</td>
<td>PB</td>
<td>3 7 25 24 7</td>
<td>59</td>
<td>41</td>
<td>61.0</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM</td>
<td>8 19 12 15 26</td>
<td>81</td>
<td>19</td>
<td>33.0</td>
<td>62</td>
</tr>
<tr>
<td>RETICULUM CELL SARCOMA</td>
<td>3</td>
<td>PB</td>
<td>3 39 41 13</td>
<td>96</td>
<td>4</td>
<td>60.0</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM</td>
<td>13 19 22 26</td>
<td>80</td>
<td>20</td>
<td>53.0</td>
<td>65</td>
</tr>
<tr>
<td>HYPOCHROMIC ANEMIA</td>
<td>4</td>
<td>PB</td>
<td>14 12</td>
<td>26</td>
<td>74</td>
<td>23.0</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM</td>
<td>10 15 2</td>
<td>27</td>
<td>73</td>
<td>15.0</td>
<td>31</td>
</tr>
<tr>
<td>LEUKOCYTOSIS CAUSE</td>
<td>5</td>
<td>PB</td>
<td>13 21 6 3</td>
<td>42</td>
<td>58</td>
<td>20.0</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM</td>
<td>4 10 6 3</td>
<td>23</td>
<td>77</td>
<td>15.0</td>
<td>29</td>
</tr>
</tbody>
</table>

*Alkaline Phosphatase activity expressed as mgs of P liberated per hour by 10⁷ leukocytes

Among other variables which may effect enzyme activity is the period of incubation of the cells in their own serum. As shown in Chart 4, when leukocytes were incubated in their own serum the enzyme activity doubled itself in about 2 hours and rise gradually to reach equilibrium between 4-6 hours.

Differences in alkaline phosphatase concentration in the leukocyte may be conditioned by (1) enzyme activity in the serum, (2) avidity of the cell for the enzyme, or (3) intrinsic control in the cell itself. In the study of this problem the lack of a relationship between serum alkaline phosphatase and leukocyte alkaline phosphatase was confirmed. Experiments were then performed using white blood cell suspensions from cases of normal subjects, of chronic myelogenous leukemia, of a case of myeloid metaplasia and of pyogenic infections. The cells were incubated for one hour at 37 C. in saline and various sera (see table 4). Following incubation alkaline phosphatase was determined biochemically in the usual manner and new blanks were run to correct for the possibility of an increase in the intracellular inorganic phosphorus. In all cases the cell alkaline phosphatase rose during incubation but this was independent of the serum or saline menstrum. These findings suggest that the cell alkaline phosphatase is not regulated in vitro by any factor in the serum but is intrinsic to the leukocyte itself.

SUMMARY

(1) In a large series of individuals alkaline phosphatase activity in the leukocyte was studied by biochemical and histochemical methods.

(2) The value of combined histochemical and biochemical measurements is demonstrated in this report.

(3) In chronic myelogenous leukemia the neutrophil contain very little alkaline phosphatase activity while in myeloid metaplasia and pyogenic infections morphologically similar cells exhibit a great increase in this enzyme.

(4) Various factors which might influence alkaline phosphatase activity in
**Table 4**

To show the effect of incubation of cells in saline, their own serum, and the serum from other cases with varying amounts of cell alkaline phosphatase.

<table>
<thead>
<tr>
<th>WBC Suspension</th>
<th>Before Incubation</th>
<th>After Incubation for One Hour in:</th>
<th>Serum Alk. Phos. in B. Units</th>
<th>Serum Inorganic P. in M.G.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td>28.8</td>
<td>Saline, C.M.L. Serum, Normal Serum, High A.R. Serum</td>
<td>45.0 3.02 2.48</td>
<td></td>
</tr>
<tr>
<td>C.M.L.</td>
<td>15.2</td>
<td>70.0 69.0 45.0</td>
<td>3.02 2.13 1.49</td>
<td></td>
</tr>
<tr>
<td>High A.R.</td>
<td>136.0</td>
<td>155.0 157.0</td>
<td>8.07 1.49</td>
<td></td>
</tr>
</tbody>
</table>

+ Chronic Myelogenous Leukemia

* High Cell Content of Alkaline Phosphatase (a case of Pyogenic Infection)
leukocytes were investigated and it was found that on incubation in serum or saline the cell alkaline phosphatase activity increased. However, this in vitro rise was unrelated to the source of the serum menstrum.

**SUMMARIO IN INTERLINGUA**

1. In un grande serie de individuos le activitate de phosphatase alcalin in leucocytos esseva studiate per methodos bio- e histochemic.

2. Le valor de combineate mesurationes histo- e biochimic es demonstrate in le presente reporto.

3. In chronic leucemia myelogene le neutrophilos ha bassissime activitate de phosphatase alcalin, durante que in metaplasia myeloide e in infectiones pyogene cellulas que es morphologicamente affin exhibi un grande augmento de ille enzyma.

4. Varie factores que es possibilmente capace a afficer le activitate de phosphatase in leucocytos esseva investigate. Il esseva constatate que incubation in sero o in solution salini resulta in un augmento del activitate cellular de phosphatase alcalin. Tamen, iste augmento in vitro esseva sin relation al origine del menstru seral.

**REFERENCES**

3 KLEIN, E.: Personal communication.
Histochemical and Biochemical Studies on Leukocyte Alkaline Phosphatase Activity

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