Comparison of Alkaline Phosphatase from Human Normal and Leukemic Leukocytes

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SUMMARY

The alkaline phosphatase of leukocytes from normal subjects, patients with chronic granulocytic leukemia, and patients with reactive granulocytosis have been characterized by means of agarose column chromatography, starch gel electrophoresis, Michaelis constant for p-nitrophenyl phosphate, heat inactivation, inhibition by L-phenylalanine, Ouchterlony double immunodiffusion precipitation, and immunoelectrophoresis using rabbit anti-leukocyte alkaline phosphatase serum. The same antisera was used to quantitate the amount of alkaline phosphatase protein in purified preparations of the enzyme obtained from leukocytes of patients with chronic granulocytic leukemia, reactive granulocytosis, and normal individuals. Purified alkaline phosphatase from all three sources had similar biophysical and biochemical characteristics, but the enzyme specific activities were shown to be different using the antisera and quantitative precipitation techniques. This finding was corroborated by using disc gel electrophoresis which showed that similar amounts of enzyme protein had markedly different enzyme activity, i.e., the enzyme from chronic granulocytic leukemic leukocytes had low specific activity, the enzyme from normal leukocytes had intermediate specific activity, and the enzyme from patients with reactive granulocytosis had a very high specific activity.

INTRODUCTION

A decreased activity of leukocyte alkaline phosphatase (EC. 3.1.3.1) (34, 36) is found in patients with CGL. 2

Elevations of leukocyte alkaline phosphatase have been reported in several conditions associated with RG (34, 36). Variations in the activity of alkaline phosphatase from the normal may be attributed to either a quantitative difference in enzyme concentration or to qualitative changes in the enzyme which could either enhance or reduce enzymatic activity. In the present study, alkaline phosphatase from normal, CGL, and RG leukocytes have been characterized by biophysical, biochemical, and immunologic techniques.

MATERIALS AND METHODS

Assay Procedures. Alkaline phosphatase was assayed at 37°C in a system consisting of 250 μmoles Tris hydrochloride, pH 10.0, 1.0 μmole magnesium chloride, 8.0 μmoles p-nitrophenyl phosphate, disodium salt (Sigma 104), and enzyme and water to a total volume of 1.0 ml. The change in optical density was followed continuously at 410 m/μ following the administration of chemotherapeutic agents.

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2Abbreviations used are: CGL, chronic granulocytic leukemia. RG, reactive granulocytosis, which is defined as any elevation of the absolute granulocyte count not due to a primary disease of the myeloid series associated with increased leukocyte alkaline phosphatase (this term is used instead of "leukemoid reaction" because some of the elevations in white cell count were not sufficient to qualify as a true "leukemoid reaction" but were associated with increased leukocyte alkaline phosphatase; also, the etiology of the increase in leukocyte number was quite varied from infections to rebounds in the white count following the administration of chemotherapeutic agents.

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9 volumes of Tris-saline solution and the centrifugation repeated. The two extracts were combined and dialyzed overnight against 0.05 M Tris, pH 7.5. After dialysis the partially purified enzyme was concentrated 20 fold by ultrafiltration using a collodion bag with a porosity of less than 15 μ (Kollodiumhülse Gottingen, supplied by Schleicher & Schuell). The nonfiltrable solution, consisting of proteins of molecular weight 30,000 and greater, contained the enzyme; this step resulted in an overall increase in alkaline phosphatase specific activity (Table 1). Some of the experiments were carried out using this partially purified enzyme. The final step in purification was accomplished by placing 0.3-ml aliquots of concentrated, partially purified enzyme on a 1 x 20 cm column of Bio-Gel 1.5 M Agarose and eluting the enzyme with 0.05 M Tris hydrochloride, pH 7.5. Table 1 illustrates representative activities, recoveries, and purification of enzymes from normal, RG, and CGL leukocytes. Purification varied from 21.6 fold for the CGL enzyme to 52.0 fold for the normal enzyme.

**Electrophoresis.** Vertical starch-gel electrophoresis was carried out at 4°C in a discontinuous borate buffer system (26), pH 8.2, for five hours at 22.5 milliamperes constant current. The enzyme preparations were n-butanol extracts which had to be redialyzed and concentrated by collodion membrane filtration. Due to low activity, CGL leukocyte extracts were routinely concentrated five fold more than the enzyme from normal leukocytes. Longitudinal gel slices were stained directly for alkaline phosphatase using α-naphthyl phosphate and fast blue BB (Borden Chemical Co.) as the coupling dye.

**Heat Stability Studies.** The stability of the various enzymes purified through n-butanol extraction, dialysis, and collodion membrane filtration was studied at fixed temperatures using a circulating water bath with automatic temperature control, and the temperature was monitored with a thermister-activated probe.

**Kinetics.** Quantitative inhibition studies were carried out on alkaline phosphatase purified through Step 2 by continuously monitoring the optical density at 410 mμ at 37°C in a Gilford spectrophotometer Model 2000. The substrate and inhibitors were dissolved in 0.165 M Tris hydrochloride, pH 10.0, containing 10⁻³ M MgCl₂. Since the inhibitors are buffers, after dissolving them in 0.165 M Tris, the pH was readjusted to 10.0. The pH was monitored throughout the reaction and did not vary more than ±0.05 pH units. Initial velocities were determined from slopes obtained during the first 10 minutes of hydrolysis, this accounted for less than 5% of the total hydrolysis. D-Phenylalanine (15 mM) was added to several other reaction mixtures as another control determination and produced velocities indistinguishable, within experimental error, from those obtained without added D-phenylalanine. Double reciprocal plots of enzyme activity versus substrate concentration were made with and without added L-phenylalanine using enzyme from all three cell types (21). The lines were determined by least squares analysis of the data, and the slopes, x intercepts, and y intercepts were determined by computer analysis using the statistical technic of Cleland (4). From these data apparent Kₘ's were determined with p-nitrophenyl phosphate as substrate.

**Molecular Weight Determination.** A measurement of the

<table>
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<th>Cell type</th>
<th>Normal</th>
<th>CGL</th>
<th>RG</th>
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</thead>
<tbody>
<tr>
<td><strong>Activity</strong></td>
<td>Fold purification</td>
<td>Yield (%)</td>
<td>Fold purification</td>
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<tr>
<td>Sonicate</td>
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<td>100</td>
<td>0.083</td>
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<td>Step 1, n-butyl alcohol extract</td>
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<td>3.4</td>
<td>36</td>
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<tr>
<td>Step 2, membrane concentrated</td>
<td>9.9</td>
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<td>46</td>
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<tr>
<td>Step 3, agarose column purified</td>
<td>18</td>
<td>52</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 1

- Purification of leukocyte alkaline phosphatase. CGL, chronic granulocytic leukemia; FG, reactive granulocytosis.
- Activity expressed as micromoles of p-nitrophenyl phosphate cleaved per 60 minutes per mg of protein.
were compared to the RG or normal preparation. The alkaline the cathodal band in the CGL leukocyte preparation as cell type were applied to the starch-gel. Two bands of activity by ultracentrifugation of these proteins in 5 to 20 percent linear sucrose density gradients by the method of Martin and Ames (22). The gradients were sampled using a gravity siphoning device, and hemoglobin was used as the standard. The runs were performed in an SW-39 head in a Spinco Model L ultracentrifuge for 5 to 9 hours for each sample. Fractions of 0.23 ml were collected and assayed for alkaline phosphatase activity.

Preparation of Antibody against Leukocyte Alkaline Phosphatase. The antigen was alkaline phosphatase obtained from the leukocytes of normal individuals and purified through Step 2. The purification of the antigen was approximately 30 fold. White New Zealand rabbits were injected subcutaneously intrascapularly with a 1:1 emulsion of enzyme (approximately 1 mg of protein) in complete Freund’s adjuvant (11). Booster injections were given at 2-week intervals for three doses, and the rabbits were bled 7 days later by cardiac puncture.

Immunchemical and Antibody-precipitation Studies. Immunodiffusion and immunoelectrophoresis were performed in agar as described by Kabat and Mayer (18). Histochemical staining of alkaline phosphatase activity in the cross-reacting band was performed using the same technics utilized for alkaline phosphatase staining of the starch-gel electrophoretogram. Antbody-precipitation studies were performed by adding 0.05 ml of antiserum to 0.05 ml of the Step 3-purified enzyme from normal, RG, and CGL leukocytes. Controls were set up by using serum obtained from rabbits prior to immunization with the enzyme. After incubation at 37°C for 1 hour and at 4°C for 16 hours, the mixtures were then centrifuged at 2500 X g for 30 minutes at 0°C and the precipitate washed three times with ice-cold saline. The precipitates were suspended in 0.10 ml saline and assayed for protein and alkaline phosphatase activity. Control tubes had no detectable precipitate or activity.

RESULTS

Kinetics. Charts 1–3 show the effect of varying substrate concentrations of the L-phenylalanine inhibition of alkaline phosphatase from leukocytes from normal individuals (Chart 1), patients with CGL (Chart 2), and patients with RG (Chart 3). The majority of the slopes of the double reciprocal plots of the inhibited reactions were parallel at substrate concentrations from 1 X 10⁻² to 1 X 10⁻⁴ M, within experimental error, indicating noncompetitive inhibition as the major mode of inhibition of all three enzymes with L-phenylalanine. Table 2 illustrates the $K_m$ values of p-nitrophenyl phosphate for the three enzymes, and they are the same within experimental error.

Electrophoresis. A vertical starch-gel electrophoretogram stained for alkaline phosphatase is illustrated in Fig. 1 and Chart 4. Approximately equal amounts of enzyme from each cell type were applied to the starch-gel. Two bands of activity were found in each sample although there was relatively less of the cathodal band in the CGL leukocyte preparation as compared to the RG or normal preparation. The alkaline phosphatase bands from all three cell types migrated approxi-

mately the same distance, suggesting no great difference in these enzymes with respect to electric charge at pH 8.2. Other starch-gel electrophoretograms using more concentrated enzyme preparations showed the slow moving isozyme with much more clarity. Treatment of the enzyme preparations with commercial neuraminidase from Vibrio cholerae by the procedure of Moss et al. (25) afforded a slight retardation of the anodal bands in all three cases (Fig. 1; Chart 4).

$S_{0.725}^{0.20}$ value for Agarose 1.5 M (Step 3)—purified normal, CGL, and RG leukocyte alkaline phosphatases was obtained by ultracentrifugation of these proteins in 5 to 20 percent linear sucrose density gradients by the method of Martin and Ames (22). The gradients were sampled using a gravity siphoning device, and hemoglobin was used as the standard. The runs were performed in an SW-39 head in a Spinco Model L ultracentrifuge for 5 to 9 hours for each sample. Fractions of 0.23 ml were collected and assayed for alkaline phosphatase activity.

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Heat Stability. n-Butanol extracts were studied at three temperatures: 48 ± 0.5, 51 ± 0.5, and 58 ± 0.5°C. At 48°C all three enzymes retained 76 ± 10% of their activity after 2 hours. At 58°C all three enzymes lost 100% of their activity after 30 minutes. At 51°C all three lost 35 ± 2% of their activity after 1 hour, 58 ± 3% of their activity after 2 hours, and 83 ± 6% of their activity after 6 hours.

Molecular Weight Determinations. Ultracentrifugation analysis of the three alkaline phosphatases purified through chromatography gave a S20,w value for CGL of 17.6 X 10⁻¹³ sec⁻¹, for the normal enzyme a value of 14.5 X 10⁻¹³ sec⁻¹, and for the RG enzyme a value of 15.8 X 10⁻¹³ sec⁻¹. The estimated molecular weights (22) using these figures are 410,000 ± 75,000 for the normal enzyme, 550,000 ± 75,000 for the CGL enzyme, and 470,000 ± 75,000 for the RG enzyme. Because of the uncertainty in the determination (± 1.5 X 10⁻¹³ sec⁻¹, for any sample) the differences between these various cell types are not considered significant. This relatively large molecular weight stands in contrast to that determined for other mammalian alkaline phosphatases, such as the calf intestinal enzyme (7) with an estimated molecular weight of 100,000 and the HeLa enzyme (M. J. Griffin, unpublished data) with an estimated molecular weight of 100,000.

Results of Immunodiffusion Studies. Fig. 2 illustrates the precipitation bands obtained when antinormal leukocyte alkaline phosphatase was placed in the center well of the immunodiffusion plate and purified normal, RG, and CGL alkaline phosphatase preparations were placed in the outer wells. A single precipitation line was formed with all three enzymes without evidence of spur formation suggesting immunologic identity of the alkaline phosphatase from all three sources.

Results of Immunoelectrophoresis. Fig. 3 illustrates the electrophoretogram on agar of normal, CGL, and RG alkaline phosphatase stained for alkaline phosphatase activity. In all of the experiments using agar electrophoresis, 15 microliters of CGL enzyme (0.8 unit/ml), 15 microliters of normal enzyme (4.6 units/ml), and 15 microliters of RG enzyme (35.8 units/ml) were placed in the sample wells. Equal amounts of CGL and normal leukocyte alkaline phosphatase protein were applied to the gel, but considerably smaller amounts of RG protein was present in the sample applied to the gel because sufficient amounts of the purified protein were not available to allow use of the enzyme at the same protein level as the other two enzymes. However, despite the lower protein concentration in the RG preparation, the greatest activity was seen in the RG enzyme and the lowest in the CGL as compared to the normal enzyme. Fig. 4 illustrates the cross-reacting bands that developed when these enzymes were allowed to diffuse against antinormal leukocyte alkaline phosphatase serum. After 5 days of diffusion, cross-reacting bands were seen between the normal and CGL enzymes and the antibody, but very little was seen with the RG enzyme at this relatively low protein concentration. The cross-reacting band seems heaviest between the CGL enzyme and the antibody despite the fact that the same amount of protein was placed in the CGL and normal wells, suggesting more cross-reacting protein in the CGL sample than in the normal one. Fig. 5 illustrates a replicate immunoelectrophoretogram stained for alkaline phosphatase activity. This figure illustrates much greater alkaline phosphatase activity in the cross-reacting band in the normal sample than in the case of the CGL enzyme despite the fact that there appeared to be a heavier protein precipitation band with CGL prior to enzyme staining. There appeared to be very little cross-reaction with the RG enzyme preparation at this protein concentration. The cross-reacting band seems heaviest between the CGL enzyme and the antibody despite the fact that the same amount of protein was placed in the CGL and normal wells, suggesting more cross-reacting protein in the CGL sample than in the normal one. Fig. 6 illustrates the same immunoelectrophoretogram stained for protein by the Amido-Schwarz stain, which appears to have a line of identity with the faster-running alkaline-phosphatase band, despite the fact that they are relatively large molecular weight stands in contrast to that determined for other mammalian alkaline phosphatases, such as the calf intestinal enzyme (7) with an estimated molecular weight of 100,000, and the HeLa enzyme (M. J. Griffin, unpublished data) with an estimated molecular weight of 100,000.

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Fig. 1. Starch-gel electrophoretogram of alkaline phosphatase from normal, CGL, and leukemoid (RG) leukocytes, stained for alkaline phosphatase activity. The origin is to the left of the photograph and the anode to the right. Preparations subjected to electrophoresis included enzyme maintained at 0°C, enzyme incubated for 60 minutes at 37°C without added neuraminidase, and enzyme incubated for 60 minutes at 37°C with Vibrio cholera neuraminidase.

Chart 4. Diagram of a starch-gel electrophoretogram of alkaline phosphatase from normal, leukemoid (RG, reactive granulocytosis), and chronic granulocytic leukemia (CGL) leukocytes. The origin is at the left edge of the diagram with the anode to the right. The amount of activity in each band is depicted by the shading. Neuraminidase produced some slowing of the most anodal band of all three types of alkaline phosphatase.

Fig. 2. Ouchterlony plate demonstrating the reaction of antinormal leukocyte alkaline phosphatase with normal, RG, and CGL leukocyte alkaline phosphatase. The center well contained 20 microliters of antinormal leukocyte serum, and outer wells contained 20 microliters each of purified (Step 3) normal (NOR), RG, and CGL alkaline phosphatase.
Fig. 3. Immunoelectrophoretogram of normal, CGL, and RG alkaline phosphatase stained for alkaline phosphatase activity after electrophoresis, but prior to reaction with the antinormal leukocyte serum. The circular wells indicate the origin.

Fig. 5. Immunoelectrophoretogram of normal, CGL, and RG alkaline phosphatase stained for alkaline phosphatase activity after reaction with antinormal leukocyte alkaline phosphatase serum. The antiserum was placed in the two longitudinal slits in the gel, and the circular wells were the sites of insertion of the enzyme preparation.

Fig. 4. Immunoelectrophoretogram of normal, CGL, and RG alkaline phosphatase after reaction with antinormal leukocyte alkaline phosphatase serum. The antiserum was placed in the two longitudinal slits in the gel, and the circular wells were the site of insertion of the enzyme preparation.

Fig. 6. Immunoelectrophoretogram of normal, CGL, and RG alkaline phosphatase stained for alkaline phosphatase activity and protein (Amido-Schwarz stain) after reaction with antinormal leukocyte alkaline phosphatase serum. The antiserum was placed in the two longitudinal slits in the gel, and the circular wells were the site of insertion of the enzyme preparation.

as there are no additional cross-reacting bands seen except for the small cross-reacting band seen close to the origin, probably representing the slower isoenzyme seen on starch-gel electrophoresis.

Results of Immunoprecipitation Studies. Table 3 illustrates the specific activity of the antigen-antibody precipitate obtained when antinormal leukocyte alkaline-phosphatase serum was added to purified alkaline phosphatase from normal, RG, and CGL leukocytes. Approximately equal amounts of enzyme protein from each cell type were mixed with an equal volume of undiluted antiserum. Immunoprecipitation experiments indicated undiluted serum was near optimal in affording maximal complex precipitate formation with quantitative recovery of enzyme activity. The specific activities are quite different in each cell type and are similar to the relative activities observed in the whole cell sonicates and the purified enzyme. For example, the antigen-antibody complex using the enzyme from CGL leukocytes had the lowest specific activities (Table 3) as did the sonicate from CGL leukocytes (Table 1). The enzyme from normal leukocytes had intermediate specific activity, and the enzyme from the RG leukocytes had the highest specific activity both in sonicates and antigen-antibody precipitate (Tables 1, 3). Experiments 1 and 2 of Table 3 are repeats of the same type of experiment using purified enzymes.
from different individuals. Further substantiation of the immunoprecipitation results was obtained from acrylamide disc gel electrophoretic analysis of the Agarose column-purified enzymes. As Fig. 7 shows, no detectable contaminating proteins appear when the most highly purified enzyme preparations are stained for both enzyme activity and protein. This indicates these preparations and essentially homogenous and, therefore, represent adequate materials for comparing enzyme specific activities.

**Effects of Zn**. Attempts were made to activate the enzyme from CGL leukocytes by the addition of Zn at 10^{-3}, 10^{-4}, and 10^{-5} M concentrations, a procedure which has been reported to activate *E. coli* apoalkaline phosphatase (31). It was found that Zn inhibited the enzyme from CGL leukocytes at 10^{-3} and 10^{-4} M concentrations and had no effect at 10^{-5} M.

Table 3

<table>
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<th>Cell type</th>
<th>Normal</th>
<th>RG</th>
<th>CGL</th>
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<tr>
<td>Experiment 1</td>
<td>0.94a</td>
<td>2.9</td>
<td>0.077</td>
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<tr>
<td>Experiment 2</td>
<td>0.34</td>
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*aSpecific activity expressed as micromoles of p-nitrophenyl phosphate cleaved per 60 minutes per mg of antigen-antibody complex.

![Disc gel electrophoretogram of purified CGL, normal (NOR), and RG alkaline phosphatase stained for protein (P) and alkaline phosphatase (AP).](image)

Fig. 7. Disc gel electrophoretogram of purified CGL, normal (NOR), and RG alkaline phosphatase stained for protein (P) and alkaline phosphatase (AP). The origin is at the anode and the anode at the bottom. (The dark band toward the anodal end of the CGL-AP gel is an artifact created by a break in the gel at that point and does not indicate a band of enzyme activity.)

**DISCUSSION**

Previous studies of human leukocyte alkaline phosphatase have been performed predominantly by histochemical technics as described by Gomori (14) and Kaplow (19). These studies have consistently shown that most CGL polymorphonuclear leukocytes had less histochemical alkaline phosphatase activity than normal cells (34). A biochemical assay was used by Valentine and Beck (36) with results similar to the histochemical observations. It has been suggested (1, 33) that the alkaline phosphatase activity in CGL leukocytes correlates with the observed loss of the long arms of chromosome 21 (Philadelphia chromosome). Another possible explanation for the decreased activity of this enzyme in this cell type is a decrease in the specific activity of the enzyme which might be due to a difference in its physical and biochemical characteristics. The present study was designed to investigate human leukocyte alkaline phosphatase from normal individuals, patients with CGL, and patients with granulocytosis, to determine whether the variation in activity could be correlated with differences in enzyme characteristics.

The $K_m$ values using p-nitrophenyl phosphate as substrate were not significantly different for alkaline phosphatase from all three cell types and were in the same range reported for HeLa alkaline phosphatase (16). L-Phenylalanine inhibited the enzyme from all three types of leukocytes in an uncompetitive manner (coupling inhibition) (35). This type of inhibition, a function of both substrate and inhibitor concentration, has been reported for rat intestinal (8, 9, 12), human placental (9), and HeLa (5) alkaline phosphatase.

Disc gel electrophoresis and immunoelectrophoresis showed substantial homogeneity of the purified alkaline phosphatase from all three cell types. The disc gel results even without the immunoprecipitation data would suggest that the specific activities of the purified enzymes reflect the activity of the enzyme within the cell and represent a reason for the differing amounts of enzyme activity characteristic of the three cell types. All three enzymes are precipitated by antibody to normal leukocyte alkaline phosphatase, and the results of double diffusion analysis suggest this complex is specific for an alkaline phosphatase enzyme-protein complex. The antigen-antibody complex of these three enzymes have markedly different specific activities. If one assumes that the ratio of antigen to antibody in the three types of enzyme-antibody complex is approximately the same, then for all three enzymes the finding of markedly different specific activities in the alkaline phosphatase from these three cell types suggests three possibilities: (a) there are variable amounts of inactive alkaline phosphatase protein in these three types of leukocytes which retain immunologic identity and dilute the active alkaline phosphatase in the antigen-antibody complex; (b) in each condition there is some alteration in all of the enzyme molecules which does not change the immunologic reactivity but does alter the specific activity of the molecule, or (c) leukocyte alkaline phosphatase is a heteropolymeric protein (aggregate) which contains varying amounts of active alkaline phosphatase protein.

Previous workers have suggested that the life span of the granulocyte might influence the level of alkaline phosphatase.
Leukocyte Alkaline Phosphatase

(17). If the enzyme was synthesized in the myelocyte prior to being released into the circulation, and if the enzyme in the circulating granulocyte loses activity without loss of enzyme protein as the cell ages, a large amount of inactive enzyme protein would be found in a relatively aged cell such as a CGL granulocyte. Mechanisms which might account for progressive loss of enzyme activity could include loss of Zn\(^{++}\) from the active site or conformational changes which affect enzyme activity. Vallee (37) demonstrated that normal leukocytes contained approximately 25 times as much zinc as red cells and that a protein could be isolated from the leukocyte that accounted for 81% of the intracellular zinc. Gibson et al. (13) demonstrated low levels of zinc in chronic granulocytic leukemic leukocytes, and elevated levels in infection have been reported (38). Trubowitz (32) demonstrated that purified human leukocyte alkaline phosphatase contained substantial amounts of zinc. These findings plus our own might suggest that the activity of the enzyme is regulated by the Zn\(^{++}\) apoenzyme ratio and that the changes in specific activity observed in the present study are due to differences in zinc content of the enzyme from the three types of leukocytes. In the present study we were unable to activate the CGL enzyme by the addition of Zn\(^{++}\). Trubowitz was able to partially reactivate leukocyte alkaline phosphatase with Zn\(^{++}\) after inactivating it with ethylenediaminetetraacetate (EDTA). Our failure to activate the alkaline phosphatase from CGL leukocytes suggests that the \textit{in vivo} enzyme differs in some way (such as configuration) from the EDTA-treated enzyme since the EDTA-treated enzyme can be at least partially reactivated while the CGL enzyme cannot. Proof of these various alternatives will only be possible after sufficient amounts of purified enzyme can be obtained to allow subunit characterization and quantitation of the Zn\(^{++}\) content of the purified enzyme from the three types of leukocytes.

Another aspect of mammalian alkaline phosphatase regulation \textit{in vivo} is suggested by the studies utilizing L-phenylalanine as a stereospecific inhibitor. Leukocyte (23, 30), HeLa (6), and mouse intestinal (24) alkaline phosphatases are induced by adrenocortical hormones and they have been shown to be inhibited by L-phenylalanine (5). On the other hand, other mammalian alkaline phosphatase activities not induced by glucocorticoids, such as kidney and fibroblast alkaline phosphatase, are relatively insensitive to inhibition by L-phenylalanine (5). Therefore, this inhibition may be a distinguishing characteristic of hormone-regulated alkaline phosphatases.

Starch-gel electrophoresis of CGL leukocyte alkaline phosphatase has been reported to give a pattern which differs from the alkaline phosphatase of normal leukocytes (20, 28, 29). The data reported in this paper show no significant difference in the electrophoretic pattern of the high level RG cells, the medium level normal cells, and the low level CGL leukocytes. The observation that neuraminidase slows the migration of the anodal bands of all three cell-type alkaline phosphatases equally (Chart 4) would suggest that there is the same amount of sialic acid on the isoenzyme from each of the three cell types. Variations in migration of leukocyte alkaline phosphatases previously reported (27) may be related to differing amounts of sialic acid residues (3). Heat stability curves carried out on enzyme from all three cell types were almost identical, indicating that the conformational resistance to irreversible denaturation by heat of the three enzymes is approximately the same.

The results described above would suggest that alterations in the level of human leukocyte alkaline phosphatase are mediated in some way through the activity of each enzyme molecule and that these changes result in marked changes in the specific activity of the enzyme molecules, but they do not markedly alter electrophoretic migration, \(K_m\) for \(p\)-nitrophenyl phosphate, molecular weight, or heat stability. It is possible that these changes could be related to increased or decreased numbers of active sites on the molecule although the possibility has not been directly tested. These results are similar to the results obtained by Griffin and Cox (16) concerning the mechanism of regulation of HeLa cell alkaline phosphatase by hydrocortisone.

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REFERENCES


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