

## Neutrophil Alkaline Phosphatase: Comparison of Enzymes From Normal Subjects and Patients With Polycythemia Vera and Chronic Myelogenous Leukemia

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To determine whether decreased alkaline phosphatase activity in the granules from neutrophils of patients with chronic myelogenous leukemia (CML) was due to an absence of enzyme or the production of defective enzyme, we compared the immunologic properties of granule alkaline phosphatase derived from patients with CML with that of normal subjects and patients with polycythemia vera (PRV). Antisera prepared in rabbits against granule alkaline phosphatase purified from the neutrophils of a patient with PRV produced a single precipitin line of antigenic identity when reacted with extracts of normal, PRV, and CML neutrophil granules. A histochemical stain for alkaline phosphatase activity (alpha-naphthyl acid phosphate coupled with Fast Blue RR) specifically stained the precipitin line. A variety of quantitative precipitin techniques failed to produce satisfactory precipitation of alkaline phosphatase activity. Comparative analyses were therefore performed by affinity chromatography using goat antirabbit-gammaglobulin linked to Sepharose 4B to adsorb alkaline

phosphatase complexed with rabbit gamma globulin. With this method, 100% of CML, normal, and PRV alkaline phosphatase could be adsorbed. Using limiting concentrations of antibody, a proportionally smaller fraction of enzyme activity was adsorbed as the concentration of PRV alkaline phosphatase or normal alkaline phosphatase was increased. Extracts of CML granules containing comparable amounts of protein but 200-fold less alkaline phosphatase activity per milligram did not specifically reduce adsorption. Thus, in CML, we found no evidence that the granulocytes contained a large amount of antigenically normal but enzymatically defective alkaline phosphatase. Examination of electron micrographs revealed no significant differences in the number or distribution of granules in the granulocytes of normal subjects or patients with PRV or CML. This suggests that the low level of neutrophil alkaline phosphatase in CML granulocytes is the result of decreased enzyme content and not a consequence of synthesis of catalytically defective enzyme.

**W**ACHSTEIN OBSERVED that neutrophil alkaline phosphatase may be markedly depressed in chronic myelogenous leukemia (CML).<sup>1</sup> This finding has been confirmed by numerous workers,<sup>2,3</sup> but no pathologic significance has been attributed to it. Alkaline phosphatase activity is almost always depressed in the neutrophils from patients with untreated CML, although it may rise following chemotherapy.<sup>4,5</sup> It is also depressed in other pathological conditions.<sup>6</sup> The frequent correlation of depressions of neutrophil alkaline

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phosphatase with CML has suggested that there may be a pathophysiologic link between the etiology of CML and the decreased activity of the neutrophil enzyme.

Alkaline phosphatase activity has been found in the specific granules of mature neutrophils.<sup>7,8</sup> Enzyme extracted from the granule-containing fraction of homogenized neutrophils has been purified to apparent homogeneity,<sup>9</sup> i.e., an antibody prepared against purified PRV alkaline phosphatase precipitated with a single determinant in crude granule extracts. No significant differences between the alkaline phosphatases purified from normal subjects and patients with polycythemia vera (PRV) were found.

In the investigations reported here, comparisons were made between the reactivity of alkaline phosphatase from granule extracts from neutrophils of normal subjects and patients with CML and PRV. Studies attempting to quantitate the interaction were of particular interest because they bear upon the question as to whether CML neutrophils have a less active or inactive, but antigenically similar, alkaline phosphatase or simply have less of an apparently normal enzyme. If antigenically cross-reacting material with low alkaline phosphatase activity were identified in the neutrophils of patients with CML, it would suggest that an abnormal enzyme were being synthesized, perhaps the result of genetic alteration. Alternatively, the presence of less, but normally active, enzyme would suggest a reduction in cellular alkaline phosphatase content.

## MATERIALS AND METHODS

### *Patients*

Patients with chronic myelogenous leukemia were newly diagnosed and untreated. Patients with polycythemia vera had been treated only with phlebotomy. Diagnoses were based on clinical criteria.<sup>10</sup> No karyotype studies were performed.

### *Analytical*

Methods of cell counting, protein determination,<sup>11</sup> and enzyme assay have been described in detail.<sup>9</sup> A unit of enzyme activity hydrolyzed 1  $\mu$ mole *p*-nitrophenyl phosphate (*p*NP) per min at 37°C when assayed in 0.54 mM *p*NP, 1.0 mM MgCl<sub>2</sub>, 1.0 M Tris, 0.12 M HCl (pH 9.0).

### *Preparation of Homogenates*

Leukocytes were freed of red blood cells by dextran sedimentation and hypotonic lysis, homogenized in 0.25 M sucrose, and separated into fractions by differential centrifugation.<sup>9</sup> The 39,000 *g* pellet of the postnuclear, 400-*g* supernatant contained the granules and 85% of the alkaline phosphatase activity.<sup>9</sup> It was extracted with sodium dodecyl sulfate (SDS) at 6 g/100 ml. Insoluble material was removed by 39,000 *g* centrifugation.

### *Immunologic Methods*

Preparation of rabbit antisera to alkaline phosphatase purified from the neutrophil granules from a patient with polycythemia vera has been described.<sup>9</sup> Sera collected from the same rabbits before immunization and from other rabbits immunized in an identical fashion against bovine IgG were also used. IgG was purified from the sera on DEAE-cellulose.<sup>12</sup>

Double diffusion precipitin analyses<sup>13</sup> were performed in 1.0% Ionagar (Oxoid Division, Consolidated Laboratories, Chicago Heights, Ill.) in 0.04 M Tris-HCl, pH 8.6, 0.1% Triton X-100, 1:10,000 merthiolate.

Alkaline phosphatase and rabbit antiserum were mixed in various proportions and incubated at 37°C or 4°C for various intervals. Quantitative separation of antigen-antibody aggregates was

attempted by centrifugation at 39,000 g, sucrose density gradient sedimentation, or membrane filtration on an Oxoid filter [N.50 (insulin immunoassay kit), Amersham/Searle Corporation, Arlington Heights, Ill.].

### Chromatographic Procedures

Affinity chromatography<sup>13</sup> was performed in 0.7 × 4-cm polypropylene columns (Bio-Rad Laboratories, Richmond, Calif.) using 1.5 ml goat antirabbit IgG-Sepharose as the adsorbent. Goat IgG with antirabbit IgG specificity was obtained from commercially available serum (Gateway Immunosera Co., Cahokia, Ill.) and purified by DEAE-cellulose chromatography.<sup>12</sup> It was covalently linked to Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.) activated with CNBr.<sup>13</sup>

The adsorbent was equilibrated in 0.04 M Tris, 0.036 M HCl, pH 7.2, 0.1% Triton X-100 (SB). Material was applied to the adsorbent by gently layering 0.4 ml on the upper surfaces. In all cases, the antigen-antibody interaction was allowed to proceed for at least 1 hr at 23°C before chromatography. Elution was performed with SB using a Harvard Pump (Harvard Apparatus, Millis, Mass.) operating at 0.21 ml/min. Fractions were collected and pooled so as to contain all of the eluted enzyme activity (1.7 ml total volume). Prior to assay, in order to maximize and stabilize activity, 0.5-ml samples were diluted with 0.4 ml of 1.0 M Tris-HCl, pH 9.0, 1.0 mM mgCl<sub>2</sub> and incubated at 23°C for 15 min and 37°C for 5 min. Without this treatment, enzyme activity appeared to increase with time, presumably a reflection of the change in pH.

### Staining Procedures

Alkaline phosphatase activity was demonstrated with  $\alpha$ -naphthyl acid phosphate and Fast Blue RR.<sup>9</sup> Protein was detected with amido black.

### Electron Microscopy

Whole leukocytes obtained from pellets of hypotonically hemolyzed samples of the supernatant fractions of dextran-sedimented whole blood were stored at 4°C for 4-6 hr. The leukocytes were fixed in an equal volume of 1.0% OsO<sub>4</sub> for 30 min, washed and dehydrated in graduated concentrations of ethanol followed by propylene oxide, and embedded in Araldite. Thin sections, stained with uranyl acetate and lead citrate, were examined and photographed with a Siemens 1-A electron microscope, using a 50- $\mu$  objective aperture and a 60KV accelerating voltage.

## RESULTS

### Extraction of Alkaline Phosphatase From Neutrophil Granules

The results of SDS treatment of granules from normal subjects and patients with CML and PRV are shown in Table 1. The specific activity of the PRV extract was 200- to 1000-fold greater than that of the CML.

Table 1. Recovery of Alkaline Phosphatase From Neutrophil Granules

Expt. No.	Diagnosis	Alkaline Phosphatase U/10 <sup>9</sup> WBC	Protein mg/10 <sup>9</sup> WBC	Specific Activity U/mg
1	CML	0.0027	8.9	0.0003
2	CML	0.0034	14.6	0.0002
3	CML	0.055	8.6	0.006
4	PRV	1.8	11.1	0.16
5	PRV	3.7	17.	0.22
6	PRV	2.6	10.9	0.23
7	Normal	0.44	19.6	0.022

The results of pNP phosphatase and Lowry protein analyses of SDS extracts of the 39,000 g pellets of postnuclear supernatants are shown. Each preparation was obtained from the neutrophils of a different subject.

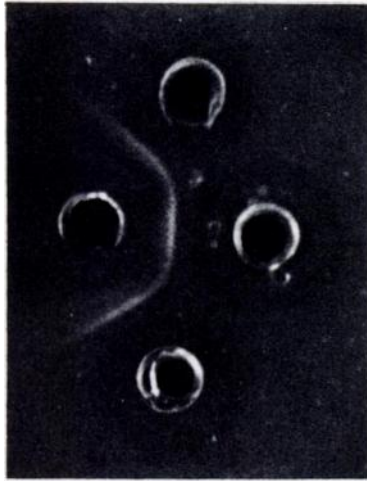


Fig. 1. Immunologic comparison of SDS extracts of CML, normal, and PRV neutrophil granules. The well on the left contained rabbit antiserum prepared against purified PRV alkaline phosphatase. In clockwise order from the top, the other wells contained SDS extracts of CML, PRV, and normal granules. The extracts had been passed through Sephadex G-25 to reduce the SDS concentration prior to immunodiffusion. A control analysis with unimmunized rabbit serum showed no precipitation.

#### *Immunologic Comparison of Granule Extracts From Normal Subjects and Patients With CML or PRV*

A photograph of a double-diffusion precipitin analysis is shown in Fig. 1. Antiserum prepared against PRV alkaline phosphatase precipitated with an antigenic material in SDS extracts of neutrophil granules. The precipitin line of the extracts from normal, PRV, and CML granules fused in a pattern of immunologic identity. Identically prepared, washed plates (not shown) stained with an histochemical stain for alkaline phosphatase activity (see Materials and Methods) showed specific staining of the precipitate.

#### *Effect of Specific Alkaline Phosphatase Antiserum on Enzymatic Activity*

Alkaline phosphatase activity was not inhibited by specific antiserum prepared against the enzyme, as shown in Table 2. The results were not affected by varying the relative concentrations of alkaline phosphatase and antibody, centrifuging the mixture at 39,000 *g* before assay, or passing it through a membrane filter. Under the conditions chosen, endogenous rabbit serum alkaline phosphatase made less than a 3% contribution to the activity in the mixture.

#### *Quantitative Adsorption of Alkaline Phosphatase Complexed With Specific Antiserum*

Sedimentation of alkaline phosphatase activity on sucrose density gradients was enhanced by the addition of rabbit antiserum to alkaline phosphatase but

Table 2. Effect of Antiserum on Alkaline Phosphatase Activity

Antiserum	Alkaline Phosphatase	Saline	Alkaline Phosphatase U/total volume
100 $\mu$ l	—	10 $\mu$ l	0.0006
—	10 $\mu$ l	100 $\mu$ l	0.023
100 $\mu$ l	10 $\mu$ l	—	0.024

The results of pNP phosphatase assays of mixtures of purified human neutrophil granule alkaline phosphatase and rabbit antiserum prepared against the enzyme.

**Table 3. Antirabbit IgG-Sepharose Adsorption of Alkaline Phosphatase Activity Bound to Rabbit Antialkaline Phosphatase**

Antiserum	Applied (U × 10 <sup>3</sup> )	Recovered (U × 10 <sup>3</sup> )	Bound (%)
1:333	3.55	0.000	100
1:500	3.53	0.49	86
1:1000	3.33	0.90	73
1:5000	3.47	2.08	40
None	3.33	3.07	8

The reaction mixture contained diluted antiserum and 36  $\mu$ g of a crude extract of neutrophil granule alkaline phosphatase in a total volume of 500  $\mu$ l. A 400- $\mu$ l sample was chromatographed on antirabbit IgG Sepharose, and simultaneous assays were performed on an aliquot of the applied sample and the column effluent. The results of pNP phosphatase assays of samples prior to (applied) and following (recovered) chromatography of the mixture are shown. The difference between the applied and recovered alkaline phosphatase activities is expressed as the per cent bound.

not by unimmunized rabbit serum. Far more efficient binding of alkaline phosphatase activity from mixtures of granule extracts and specific antibody was obtained by performing chromatography on antirabbit IgG-Sepharose. As shown in Table 3, a 1:333 dilution of antiserum bound 100% of the alkaline phosphatase activity to the adsorbent. At a 1:5000 dilution of antiserum, 40% of the alkaline phosphatase activity was bound.

Alkaline phosphatase adsorption was measured in the presence of unimmunized rabbit serum and in the presence of potent rabbit antiovine IgG. No adsorption was observed.

The results of chromatography of neutrophil granule extracts and rabbit anti-alkaline phosphatase on antirabbit IgG-Sepharose are shown in Fig. 2. At a fixed concentration of antiserum, binding of alkaline phosphatase activity was proportional to the concentration of enzyme applied to the column. Binding kinetics of CML alkaline phosphatase were similar to those for PRV and normal alkaline phosphatase but, because of its low specific activity, the highest concentration of CML enzyme which could be tested was less than 10% of the maximum concentrations of the normal and PRV enzyme.

To test for the presence in CML extracts of material which was antigenically similar to normal or PRV alkaline phosphatase, various amounts of granule extracts were added to a constant amount of PRV extract mixed with a constant amount of rabbit antiserum to alkaline phosphatase, as shown in Fig. 3. The amount of antibody chosen bound 57% of the PRV alkaline phosphatase ac-

**Fig. 2. Chromatography of neutrophil granule extracts and rabbit anti-alkaline phosphatase on antirabbit IgG-Sepharose.** A concentration of antiserum was chosen which bound 60% of the lowest concentration of alkaline phosphatase activity used. CML ( $\Delta$ ), normal ( $\circ$ ), and PRV ( $\times$ ) granule extracts were each mixed with antiserum and chromatographed separately. Data were derived as described in Table 3 and are expressed as units bound.

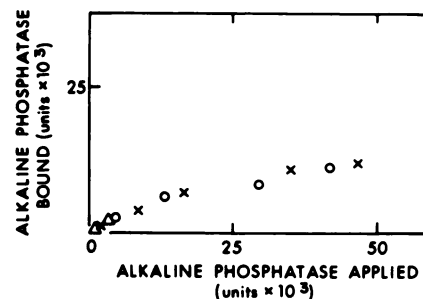
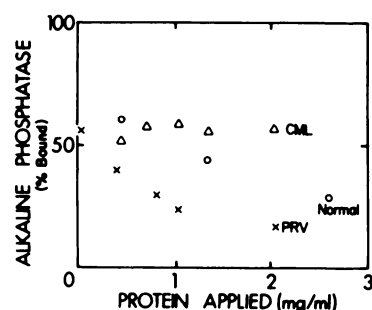


Fig. 3. Effect of increasing concentration of neutrophil granule extracts on specific binding of alkaline phosphatase complexed with specific antiserum to anti-rabbit IgG-Sepharose. A constant amount of diluted antiserum was mixed with a variable amount of granule extract from the indicated source and chromatographed. Data were derived as in Table 3.



tivity at a protein concentration of 50  $\mu$ g of PRV granule extract per ml. As the enzyme concentration was increased, progressively smaller fractions of enzyme activity were bound to the adsorbent. At a protein concentration of 2 mg of PRV granule extract per ml, only 17% of the alkaline phosphatase was bound to the adsorbent. When normal granule extract was added instead of PRV granule extract, 29% of the activity was bound to the adsorbent at a protein concentration of 2.6 mg/ml. No interference with binding by the CML granule extract was observed with concentrations as high as 2 mg/ml. Thus, there was no evidence of a substantial concentration in the CML extracts of cross-reacting material which was antigenically similar to PRV or normal alkaline phosphatase. Similar results (not shown) have been obtained with extracts from the neutrophils of two other CML patients.

#### *Electron Microscopy of Whole Neutrophils*

The primary and secondary (specific) granule content of whole neutrophils was determined by electron microscopy of preparations of leukocytes of normal subjects and patients with CML and PRV. Primary granules were identified by their larger size and marked electron density. The remainder of the granules were classified as secondary granules. Examination of more than 50 granulocytes from each subject failed to reveal any mature polymorphonuclear leukocytes which lacked specific granules.

#### DISCUSSION

The difference in alkaline phosphatase content of neutrophils from normal subjects and patients with CML and PRV has been confirmed in these studies of the granule fractions of leukocyte homogenates. Although the specific activity of the alkaline phosphatase extracted from the granules of individual patients fluctuated, we consistently observed that the recovery of alkaline phosphatase from PRV neutrophils was at least two orders of magnitude greater than the recovery from CML neutrophils.

Robinson et al.<sup>15</sup> have noted isozymes of alkaline phosphatase on starch-gel electrophoresis. As noted previously,<sup>9</sup> we have identified only one active entity by a variety of techniques in SDS extracts of neutrophil granules, although we have not subjected them to starch-gel electrophoresis.

Bottomley et al.<sup>16</sup> observed two leukocyte alkaline phosphatase isozymes on starch-gel electrophoresis. However, alkaline phosphatase behaved as a single entity under other conditions of study, including polyacrylamide-gel electro-

phoresis, sucrose density gradient centrifugation, and ion-exchange chromatography. Immunologic and biochemical comparisons of alkaline phosphatase isolated from the leukocytes of normal subjects and patients with chronic myelogenous leukemia and reactive granulocytosis showed only a single difference: CML alkaline phosphatase had a low specific activity, and reactive granulocytosis alkaline phosphatase had a high specific activity. This suggested that there might be a qualitative difference in the alkaline phosphatases and that CML leukocytes contained a relatively large amount of enzyme with low activity.

In our previously reported studies,<sup>9</sup> the specific activity of purified neutrophil granule alkaline phosphatase was seven- to tenfold greater than that reported by Bottomley et al., and the molecular weight, determined by the same method which they used,<sup>17</sup> was 160,000. The methods of extraction, the use of granule fractions instead of whole leukocytes, and the purification method chosen may account for these differences.

Previously we found no significant differences between normal and PRV enzyme by centrifugation on a sucrose density gradient, polyacrylamide-gel electrophoresis, ion-exchange chromatography, kinetic analysis, and immunodiffusion. There was insufficient CML alkaline phosphatase available to make all of these comparisons, although it is apparently identical to PRV alkaline phosphatase by qualitative and quantitative immunologic methods. The studies reported here show no apparent differences in the precipitin pattern obtained when extracts of neutrophil granules from normal subjects and patients with CML and PRV were examined by double-diffusion against rabbit antiserum to neutrophil alkaline phosphatase. The apparent paradox that rabbit antiserum causes precipitation of alkaline phosphatase activity in double-diffusion agar plates but fails to precipitate activity under the same conditions from liquid medium is unresolved. Possible explanations include coprecipitation of non-alkaline phosphatase protein by the antiserum in agar, inefficient precipitation of alkaline phosphatase in liquid medium, and the intriguing but unsubstantiated possibility that human neutrophil granule alkaline phosphatase contains multiple identical sites in appropriate proximity to produce bivalent antibody binding (as discussed in reference 18).

Antigenic identity of normal and PRV neutrophil granule alkaline phosphatase is suggested by the affinity chromatography data. The low specific activity of the CML enzyme precludes a definitive judgment on antigenic identity, although the adsorption data are consistent with it. CML neutrophil granules did not contain antigenically cross-reacting material in concentrations comparable to that in normal or PRV neutrophil granules, as judged by interference with specific adsorption of complexes of alkaline phosphatase and specific antiserum. Thus, a decreased content of "normal" alkaline phosphatase in CML neutrophils could account for all of the observations.

Morphologic estimation of neutrophil granule contents failed to reveal a difference between normal and CML or PRV granule populations. We did not confirm the report by Bessis<sup>19</sup> that the majority of mature neutrophils from patients with CML are markedly deficient in granules, despite the fact that our patients did exhibit marked reduction in alkaline phosphatase activity. Work in

progress in our laboratory suggests that the major electrophoretically separable protein components of specific granules from normal, CML, and PRV granules are identical. In this system alkaline phosphatase is not visualized as a major component.

These studies suggest that the decrease in alkaline phosphatase activity in neutrophils from patients with untreated CML is the consequence of a low content of normally active enzyme rather than the presence of structurally defective enzyme. Possible explanations for low cellular alkaline phosphatase content are the following: (1) Enhanced degradation of alkaline phosphatase either because of enzyme or specific granule instability, (2) impaired alkaline phosphatase synthesis, or (3) abnormal specific granule formation. An intriguing possibility is that normal specific granule formation is impaired as a consequence of the same process that promotes release of immature cells from the bone marrow. The observation that normal levels of neutrophil alkaline phosphatase may return as patients enter remission is consistent with this. Thus, even the abnormal clone of proliferating cells in CML is apparently capable of making normal amounts of alkaline phosphatase under certain conditions.<sup>20</sup>

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