Determination of Acid Phosphatase Activity in Normal Human Lymphocytes

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ABSTRACT

Acid phosphatase levels in pure lymphocytes from normal individuals were determined cytochemically on intact cells and spectrophotometrically on cell extracts. Good correlation was demonstrated between the results obtained with both methods. Biochemical assay showed that a normal range of 7.2 ± 0.9 and 7.1 ± 1.0 mMoles per 10^6 lymphocytes was obtained without and with tartrate inhibition, respectively. A significant loss of acid phosphatase level in the extract was found on storage. Seventy-five, 49 and 45 percent of the acid phosphatase activity remained after 3.5, 24, and 48 hours refrigeration at 4°C, with 51 percent and 32 percent after 24 and 48 hours refrigeration at −20°C, respectively. The spectrophotometric assay is less variable than the cytochemical stain. The widely utilized cytochemical method, although less reproducible, allows for evaluation of intracellular distribution of the enzyme in addition to level of activity.

Introduction

Acid phosphatase, a lysosomal enzyme, has been demonstrated in human lymphocytes.6,7,8,10,14 The association of acid phosphatase with lymphocyte subpopulations in normal and pathological states has been studied.

Cytochemical studies have shown the positivity of acid phosphatase in the leukemic lymphocytes of T-cell acute lymphocytic leukemia. Cytochemical studies have demonstrated decreased amounts of acid phosphatase in the cells of B-cell chronic lymphocytic leukemia.7,8 The degree and patterns of acid phosphatase positivity demonstrated cytochemically in normal T and B lymphocytes exhibit no significant differences. However, biochemical assays have shown higher levels of acid phosphatase activity in normal B lymphocytes than in normal T lymphocytes.8 In order to assess the clinical significance of this enzyme, the differences in methods must be studied. By using pure lymphocyte preparations from normal individuals, this investigation undertakes to compare acid

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phosphatase activity determined by biochemical and cytochemical methods. The effects of different anticoagulants and storage on the enzyme activity have also been assessed.

Materials and Methods

Reagents

Lymphocyte Separating Reagent (iron particles suspension).†

Sodium Chloride, 0.9 percent. This was prepared by dissolving 9.0 grams of reagent grade sodium chloride in one liter of distilled water.

Dextran, 6.0 percent. This was prepared by dissolving 6.0 grams of reagent grade dextran (average mol. wt. 170,000) in 100 ml of 0.9 percent saline.

Ristocetin.‡ A solution of 0.1 ml of 30 mg per ml ristocetin solution to 1 ml of plasma is used.

Ficoll, 9.0 percent. This was prepared by dissolving 9.0 grams of ficoll (mol. wt., approximately 400,000) in 100 ml of distilled water.

Hypaque, 33.3 percent. This was prepared by diluting 20 ml of 75 percent hypaque-M with 25 ml of distilled water.

Ficoll-Hypaque Solution. This was prepared by mixing 100 ml of 9.0 percent ficoll with 41.7 ml of the 33.3 percent hypaque solution.

Citrate Buffer, 0.09 M, pH 4.85. This was prepared by dissolving 18.91 grams of citric acid monohydrate in 900 ml of distilled water. The pH was adjusted to 4.85 with 1 N NaOH, and the final volume was brought up to one liter.

Tartrate-Citrate Buffer, 0.04 M tartrate, and 0.09 M citrate, pH 4.85. This was prepared by dissolving 1.5 grams of L(+)tartric acid in 250 ml of citrate buffer pH 4.85.

Stock Substrate, 15.2 mM per l. This was prepared by dissolving 0.2 grams of p-nitrophenylphosphate, disodium salt in 50 ml of distilled water.

Buffered Substrates. This was prepared by mixing one part of stock substrate with one part of citrate buffer and/or one part of tartrate citrate buffer and stored in 5 ml aliquots at −20°C.

Sodium Hydroxide, 0.1 N. This was prepared by diluting 10 ml of stock 1 N NaOH to 100 ml with distilled water.

Stock Standard of p-Nitrophenol, 10 mM. This was prepared by dissolving 0.3479 grams of pure crystals in 250 ml of distilled water.

Working Standards of p-Nitrophenol, 0, 0.25, 0.50, 1.0, and 2.0 mM per L. This was prepared by diluting 0, 0.25, 1.0, and 2.0 ml of the stock standard to 10 ml with citrate buffer pH 4.85. This was prepared fresh as needed.

Spectrophotometer. Beckman model B was used with one ml cuvets.

Lymphocyte Separation Procedure

Five ml of venous blood samples were collected in three vacutainers containing citrate, ethylene diamine tetracetic acid (EDTA), and heparin, respectively. The sample was mixed with one ml of 6 percent dextran in saline and allowed to stand for 45 minutes at room temperature for red cell sedimentation. The plasma supernatant, rich in leukocytes and devoid of platelets, was transferred to another plastic tube and treated with ristocetin. The mixture was gently agitated for 30 to 60 seconds till a "snowstorm effect" was evident, as described by Brinkhous and co-workers.‡ The mixture was then centrifuged for two minutes at 110 × g to speed platelet sedimentation. The leukocyte, rich in plasma, was decanted into another plastic tube and was then carefully layered on the top of 1.5 ml of Ficoll-Hypaque solution.† This was centrifuged at 400 × g for 30 minutes at 25°C. Mononuclear cells formed a band at the interphase right above the Ficoll-

† Technicon Instruments Corp., Tarrytown, NY.
‡ Sigma Chemicals, St. Louis, MO.
Hypaque solution, while erythrocytes and granulocytes settled to the bottom of the tube. The interphase layer was transferred to another plastic tube and treated with lymphocyte separator reagent\textsuperscript{10} which contains iron particles at 37°C for 30 minutes. The suspension was centrifuged over Ficoll-Hypaque. The interface layer consisting mainly of lymphocytes was transferred to a properly labeled tube, and diluted to one ml with saline. After a cell count was performed,\textsuperscript{9} 0.3 ml was withdrawn for cytochemical studies, and the remainder of the cell suspension was processed for spectrophotometric analysis.

**Cytochemical Procedure**

Cytochemical localization of acid phosphatase was performed\textsuperscript{12} on films.\textsuperscript{f} Acid phosphatase activity was scored as 0 for non-reactive cells, 1+ for one to two positive granules, 2+ for two to six positive granules, and 3+ for more than six granules. Total score was determined by Kaplow’s method of summing the scores assigned to 100 cells.\textsuperscript{9}

**Spectrophotometric Procedure**

The spectrophotometric procedure was a modified version of a standard procedure for determination of acid phosphatase activity using p-nitrophenylphosphate (PNPP) as substrate.\textsuperscript{6} The procedure was performed as follows: The lymphocyte suspension remaining after cell counting was centrifuged at 1900 \( \times \) g. The supernatant was discarded, and the sides of the tube were wiped. Extraction of acid phosphatase from the lymphocytes was accomplished by adding 100 \( \mu \)l of citrate buffer, pH 4.9, followed by 25 \( \mu \)l of 25 percent Triton X-100 in saline. The mixture was agitated gently for a minute and then was diluted to 500 \( \mu \)l with citrate buffer.

Acid phosphatase activity of the resulting extract was determined by incubating 200 \( \mu \)l of the citrate-buffered substrate with 20 \( \mu \)l of sample extract for 30 minutes at 37°C. Tartrate-resistant acid phosphatase activity was assayed by incubating 200 \( \mu \)l tartrate-citrate buffered substrate with 20 \( \mu \)l of sample extract simultaneously under the same conditions. Then one ml of 0.1 N NaOH was added to each incubated extract-substrate, and the absorbance read at 405 nm on Beckman Model B spectrophotometer after zeroing with a reagent blank. P-nitrophenol standards, prepared from the stock standard and treated in the same way as sample extracts, were used for preparation of a standard curve correlating concentration (mM/l) and absorbance.

Concentration expressed as mM per 10\(^6\) lymphocytes was calculated by dividing the extraction volume by the sample volume, then by the number of lymphocytes used, and then multiplying by the concentration in mM read from the reference curve.

**Results**

A pure lymphocyte suspension was obtained by first aggregating platelets with ristocetin and then utilizing the separation method described by Poon and co-workers.\textsuperscript{10} From five ml of citrated blood, 0.91 to 3.9 \( \times \) 10\(^6\) lymphocytes were obtained. The purity was 96.1 \( \pm \) 2.4 percent.

A normal range of acid phosphatase activity in pure lymphocyte extracts determined by the spectrophotometric procedure on 10 healthy males and 10 healthy females was 5.4 to 8.7 mM per 10\(^6\) lymphocytes. The overall mean acid phosphatase value was 7.2 \( \pm \) 0.9 mM per 10\(^6\) lymphocytes without tartrate inhibition and 7.1 \( \pm \) 0.10 mM per 10\(^6\) lymphocytes on incubation with tartrate containing

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* A Coulter Counter, Model ZBI, was used.
† This was prepared on a Shandon Elliott cytocentrifuge using the Sigma Chemical Co. Kit.
buffer. The enzyme activity was similar to both sexes. Repeated determinations, starting with fresh blood samples from the same individuals on three different days, were also performed; the means were 7.3 ± 1.0, 7.2 ± 1.2, and 7.0 ± 2.7 mM per 10^6 lymphocytes.

Cytochemical scoring of the acid phosphatase activity gave an overall mean of 133.6 ± 12.6. The scorings done on the samples on three different days from the same individual were similar, the mean being 144.8 ± 22.1, 125.4 ± 15.3, and 130.8 ± 22.1. The acid phosphatase activity scored by cytochemical method was completely inhibited in the presence of tartrate.

The effect of anticoagulants on acid phosphatase in lymphocytes was examined by the spectrophotometric procedure. Citrated, EDTA-anticoagulated, and heparinized blood from the same subject showed 5.8 ± 0.5, 6.2 ± 0.3, and 5.9 ± 0.1 mM acid phosphatase activity in 1 × 10^6 lymphocytes.

The stability of the lymphocyte extracts was examined 3.5, 24, and 48 hours after preparation. Samples were stored at 4°C and at −20°C. On storage at 4°C, 75 percent preservation of acid phosphatase concentration was obtained for the first 3.5 hours, and preservation of 49.4 and 45.0 percent after 24 and 48 hours. The percentage preservation on storage at −20°C was 75.2, 50.7, and 31.9 for 3.5, 24, and 48 hours, respectively.

Discussion

Acid phosphatase levels in pure lymphocytes were quantitated cytochemically on intact cells and spectrophotometrically on cell extracts so as to provide more detailed information regarding the enzyme level in health and possible relationships to lymphocyte function. Such an undertaking necessitated the establishment of a normal range for the enzyme. The availability of a pure lymphocyte suspension was imperative. An adaptation of Vives's procedure to produce pure lymphocyte suspensions was futile as the cell morphology was poor, and the results were not reproducible. Hence, ristocetin was used in the present study at a ratio of 0.1 ml of 30 mg per ml ristocetin to one ml of plasma. At this concentration, the platelets were removed without seriously affecting the morphology of the cells. Also, removal of contaminating granulocytes and monocytes was accomplished by using iron particles. Utilizing this method, an average of 96 percent lymphocytes was obtained.

Extraction of acid phosphatase from lymphocytes was accomplished with Triton X-100, as sonication appeared to denature some of the enzyme (unpublished data). Also, the use of citrate buffer pH 4.9 in place of saline seemed to yield reproducible, as well as higher acid phosphatase levels in lymphocyte extracts (unpublished data).

To study the stability of the enzyme, acid phosphatase levels were determined immediately following extraction and 3.5, 24, and 48 hours following extraction. The results demonstrated that freezing and thawing inactivates acid phosphatase.

The effect on anticoagulants on acid phosphatase activity was investigated for citrate, EDTA, and heparin. No significant differences were found.

Tartrate inhibition of the lymphocyte acid phosphatase levels determined by the spectrophotometric method was not significant. Analysis of acid phosphatase activity by cytochemical technique disclosed complete inhibition of the enzyme by tartrate. The discrepancy in the results of tartrate inhibition by cytochemical and biochemical methods is not clearly understood at this time.

Quantitation of acid phosphatase activity by cytochemical methods is less reproducible and exhibits variability. However, the more easily performed cytochemical procedure has been widely
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employed, particularly as one of several aids for evaluation and subclassification of acute leukemias.3,4 Cytochemical stains also allow assessment of the localization and patterns of distribution of acid phosphatase within cells and evaluation of cell to cell variation. This ability is particularly desirable when a number of lymphocyte subpopulations are present in significant percentages, such as in healthy individuals and in many reactive processes. The greater precision of the biochemical assay may be more helpful when a single lymphocyte subpopulation or a clone of lymphocyte which is seen in acute lymphocytic leukemia.

Previously reported results of cytochemical demonstrations of acid phosphatase activity in normal lymphocytes are in agreement with our findings.6 This method is not applicable to leukemic individuals whose platelets were unresponsive to ristocetin, in patients on high dose penicillin therapy, or in patients with von Willebrand's disease or Bernard Soudier syndrome whose platelets do not agglutinate with ristocetin.

References

12. SIGMA TECHNICAL BULLETIN, NO. 386. Revised January 1979, for cytologic demonstration of acid phosphatase in leukocytes.