Nonspecific Esterase of B Lymphocytes From a Case of Chronic Lymphocytic Leukemia and of Normal T Lymphocytes: Similar Constellations of Isoenzymes

By Joseph Yournos, Peter Burkart, Walter Mastropaolo, and Anthony Tartaglia

Lymphocytes from a case of B-cell chronic lymphocytic leukemia (CLL) were obtained in a highly purified state from a therapeutic leukapheresis preparation. The CLL lymphocytes showed a fine, scattered, granular pattern of nonspecific esterase cytochemical reactivity with either α-naphthyl acetate (αNA) or α-naphthyl butyrate (αNB) substrate as opposed to the more focal pattern of control (T) lymphocytes. Nonspecific esterase of CLL lymphocytes and normal control lymphocytes was equally resistant to inhibition by fluoride ion. Extractable nonspecific esterases from the CLL lymphocytes and from purified normal T lymphocytes were indistinguishable in regard to specific activity, substrate specificity, pH optima, and zymogram profiles on polyacrylamide gel electrophoresis at pH 9.5 and pH 4.0. Zymograms of αNA esterase and αNB esterase prepared by isoelectric focusing were also similar, with no unequivocal differences. These results are consistent with recent reports that B lymphocytes contain detectable nonspecific esterase and suggest that the B lymphocytes from this case of CLL contained a constellation of isoenzymes similar to that of normal T lymphocytes. This is interpreted as a reflection of the close kinship of these cells.

SIGNIFICANT NONSPECIFIC ESTERASE reactivity of B lymphocytes has only very recently been recognized, both cytochemically and enzymologically. Here we report a case of typical B-cell chronic lymphocytic leukemia (CLL) where lymphocytes display a fine granular pattern of nonspecific esterase reactivity. These results are in agreement with those of a previous study of CLL using different conditions of fixation and enzyme reaction. To study lymphocyte differentiation, the nonspecific esterases of the CLL lymphocytes and of normal T lymphocytes were compared both cytochemically and enzymologically.

MATERIALS AND METHODS

CLL lymphocytes were purified by dextran sedimentation and Ficoll-Hypaque density-gradient centrifugation from therapeutic leukapheresis preparations. Surface-marker analysis of purified lymphocytes included determination of sheep red blood cell receptors and of surface immunoglobulin with polyvalent fluoresceinated goat anti-human immunoglobulin.

For studies of the cytochemistry of nonspecific esterase, smears were fixed according to the Sigma modification of the procedure described by Jankila et al for acid phosphatase. Air-dried smears were fixed at room temperature for 30 sec in 38 mM sodium citrate (pH 5.8)-acetone-methanol (4:6:1). The reaction mixture for nonspecific esterase was based on the Sigma Technical Bulletin, with the following modifications. (A) Working solutions of α-naphthyl acetate (αNA) or α-naphthyl butyrate (αNB) were prepared at a concentration of 10 mg/ml in ethylene glycol monomethyl ether, stored at −20ºC and used as required. No loss of substrate potency was noted over a period of several months. (B) Reaction mixtures were buffered with 20 mM n-morpholinesulfonic acid (MES), pH 6.4. (C) A fast garnet GBC coupler was added to 20 mg/50 ml buffer. In some cases, sodium fluoride was added to the reaction mixture to a final concentration of 40 mM. Freshly prepared substrate mixture was allowed to mix 10 min at room temperature on a magnetic stirrer and filtered through Whatman no. 3 paper to remove precipitate. Cytochemical reactions were carried out for 15 min at 37ºC. Reacted smears were counterstained with methyl green solution or acid hematoxylin for 10 min at room temperature.

The procedures for purification of normal control leukocytes, enzyme extraction, and polyacrylamide slab gel electrophoresis of extracts at pH 9.5 have been presented in detail elsewhere, as has the procedure for continuous spectrophotometric assay of nonspecific esterase. Polyacrylamide slab gel electrophoresis of extracts at pH 4.0 was performed according to Li et al., except that detergent was omitted from the gel.

Isoelectric focusing of esterases was performed with an LKB-Multiphor IEF unit (LKB Instruments, Hicksville, N.Y.). Freshly prepared 5% polyacrylamide gels containing LKB ampholytes (no. 1800-101, pH range 3.5-9.5) were used. Aliquots (20–25 µl) of leukocyte extract containing approximately 2.5 µg of αNA or αNB activity were pipetted onto Miracloth strips positioned either at midgel or toward the alkaline extremity. Runs generally required 2–2.5 hr to reach the end point of 1200–1400 V at 20 mA. Gels were stained directly with αNA or αNB substrate, prepared as previously described. The linear pH gradient established by isoelectric focusing was confirmed by pH determination of extracts of gel segments and by running protein standards (Pharmacia Fine Chemicals, Piscataway, N.J.).

CASE HISTORY

The patient, a 50-yr-old white male, was initially diagnosed to have CLL 3 yr previously. His disease had become rapidly progressive and resistant to two standard chemotherapy regimens. His hematocrit was 23, WBC was 480 x 10³/liter, 98% lymphocytes, and platelet count was 14 x 10³/liter. He underwent repeated leukapheresis to stimulate active cell division and to make the cells more sensitive to cycle-specific agents. Peripheral blood smears (EDTA bloods) of the patient were examined cytochemically from this time onward.

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RESULTS

In this case of typical B-cell CLL, lymphocytes accounted for 98% or more of the peripheral blood leukocytes on all three occasions when blood was examined over 7 m. Surface marker analysis revealed that 90%–95% reacted weakly for surface immunoglobulin (polyvalent antiserum) and that fewer than 5%–10% formed rosettes with sheep erythrocytes.

Nonspecific esterase cytochemical studies of peripheral smears on these three occasions revealed a fine, scattered, granular pattern of reactivity in 45%–90% of the lymphocytes (200–500 cell count) (Fig. 1). Cells showing the intense focal reactivity associated with T lymphocytes comprised only 1.5% or less, while cytochemically negative cells amounted to 10%–55% of the total. The fine granular pattern of reactivity was observed with either αNA or αNB as substrate. The reactivity gradually diminished to undetectable in

Fig. 1. Nonspecific esterase of CLL cells with αNA, pH 6.4, acid hematoxylin (160×). In this smear of peripheral blood, about 80% of the lymphocytes show fine, scattered, granular activity. A large platelet with scattered granular activity is seen at left center. Bottom insets: An occasional cell with intense focal reactivity, presumably a T lymphocyte, and a rare monocyte with strong scattered granular activity provide a contrast in reaction pattern.

unfixed smears over several months storage at room temperature. While the majority of the lymphocytes of normal control smears was presumably of T cell type, there was a graded pattern of reactivity among cytochemically positive lymphocytes, from clearly focal or polar (15%–50%) to granular (30%–55%). The pattern of cytochemical reactivity of the purified T-cell preparations used in the study (85%–90% rosetting) ranged from focal (30%–50%) to granular (40%–60%). Monocytes on control slides and the scattered monocytes on CLL slides showed the expected intense cytochemical reactivity under all the above conditions. Fluoride ion only partially inhibited αNa and αNB esterase reactivities of CLL lymphocytes and control lymphocytes, while the intense monocytic activity on control slides was obliterated.

Nonspecific esterase activities were of similar magnitude in extracts of CLL B lymphocytes (>99% lymphocytes) and purified normal T lymphocytes (>99% lymphocytes) (Table 1). The activity of the extractable esterase of monocytes was several fold higher and showed a decided preference for αNB over αNA as substrate. Monocytes displayed a greater range of specific activity than did the lymphocytes. These values for each cell type compare well to those of Radzum et al. who used a different assay procedure.4 The extractable esterase activities of CLL B lymphocytes and normal T lymphocytes showed similar pH-esterase activity profiles (Fig. 2). Whereas αNB activity was largely unchanged over the pH range of 5.4–8.0, αNA activity of each cell type showed a marked diminution from pH 7.0 to 5.4. Control esterase from a monocyte preparation showed relatively flat curves in the same pH range.

We have previously described similar esterase zymograms of B lymphocytes from this case of CLL and normal T lymphocytes prepared by polyacrylamide slab gel electrophoresis at pH 9.5.12 Each cell type showed αNA-reactive species A1 and A2 and an αNB-reactive species B1, each partially resistant to inhibition by 40 mM fluoride ion. Figure 3 shows

![Figure 1](https://example.com/figure1.jpg)

![Figure 2](https://example.com/figure2.jpg)

![Figure 3](https://example.com/figure3.jpg)

Table 1. Specific Activity of Extractable Nonspecific Esterase From Purified Cell Preparations

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Preparation</th>
<th>mU Esterase x 10^3 per Cell</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>αNB</td>
</tr>
<tr>
<td>Monocyte</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>131</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.7</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>1</td>
<td>2.4</td>
</tr>
</tbody>
</table>

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Fig. 2. Nonspecific esterase activity of purified cell extracts as a function of reaction pH. Esterase activity of extracts was determined with 10 mM MES buffer in the pH range 5.4-7.0 (closed symbols) and with 10 mM sodium phosphate buffer in the pH range 7.0-8.0 (open symbols). Extracts (5 μl) from the following sources were assayed in duplicate: normal T lymphocytes (○●), CLL B lymphocytes (▲△), normal monocytes (■□).

Zymograms of αNA esterase and αNB esterase from CLL B lymphocytes and two preparations of normal T lymphocytes prepared simultaneously by isoelectric focusing were also similar (Fig. 4). Each αNA esterase preparation showed a widely spaced series of 20–25 isoenzymes with an isoelectric point in the range from about 4.5 to pH 8.0. A few quantitative differences were apparent, with no unequivocal relation to lymphocyte type. Zymograms of lymphocyte αNB esterase were likewise highly similar, each showing a weaker reactivity, limited to the neutral and acidic species. Zymograms of αNA esterase and αNB esterase from control monocytes were characterized by a closely spaced prominent series of isoenzymes with an isoelectric point around pH 6.0–6.5, as well as a few weaker species with isoelectric points from pH 4.0 to 6.0 and pH 7.0 to 7.5.

DISCUSSION

Nonspecific esterase chemistry has been used over the years primarily to identify monocytoid cells.11-16 A large proportion of normal peripheral T lymphocytes demonstrates a characteristic focal (Golgi-associated) alpha-naphthyl acetate esterase or alpha-naphthyl butyrate esterase.17-19 The nonspecific esterase reactivity of T cells is resistant to inhibition by fluoride ion.2

Recent reports show significant cytochemical positivity in normal B lymphocytes20 and in malignant B lymphocytes.2 In the analysis of Higgy et al.,3 lymphocytes from 4 of 9 cases of typical B-cell CLL showed fine granular positivity for αNB esterase. Our results are in agreement with the findings of Higgy et al.3 and further suggest that the reactivity of B lymphocytes in this case of CLL, like that of normal T lymphocytes, can be viewed both as an αNA esterase and an αNB esterase, which is similarly resistant to fluoride inhibition.

Reports are now appearing of nonspecific esterase activity in extracts of B lymphocytes.4,12 In view of the comparable levels of extractable nonspecific esterase in T lymphocytes and CLL B lymphocytes shown here, and previously in T lymphocytes and tonsillar B lymphocytes,4 the general observation of insignificant cytochemical reactivity in B lymphocytes appears enigmatic. This may reflect methodological differences, the difficulty of scoring a minor population of cells in normal bloods, or an overdifuse distribution of relatively modest esterase activity in B cells as opposed to the more concentrated distribution of T lymphocytes.22

Except for overall pattern of cytochemical reactivity, we have detected no other cytochemical differences nor any unequivocal enzymologic differences between nonspecific esterases of normal T cells and B cells of this case of CLL. It remains to be established if any of
the few apparent differences on isoelectric focusing represent bona fide and reproducible T-cell markers or B-cell markers. Therefore, we conclude that the constellations of nonspecific esterases from this case of CLL lymphocytes and normal T lymphocytes are highly similar, if not identical.

Radzun et al.⁴ reported isoelectric focusing zymograms of tonsillar B lymphocytes that showed only a series of αNA esterase bands with an isoelectric point from about pH 5.9 to 6.4, somewhat like monocyte zymograms. T-lymphocyte zymograms contained several additional αNA esterases. This is in contrast to our results in which zymograms of nonspecific esterase of normal T lymphocytes and CLL B lymphocytes both basically resemble the T-cell zymograms reported by Radzun et al. A possible explanation for the discrepancy between our results and those of Radzun et al. is the source of B lymphocytes. At present, we do not know whether CLL B lymphocytes or tonsillar B lymphocytes more closely represent normal circulating B lymphocytes with respect to nonspecific esterases. To our knowledge, no laboratory has yet succeeded in isolating normal circulating B lymphocytes in sufficient purity and quantity for isoenzyme analysis.

Zymogram analysis appears capable of providing highly characteristic “fingerprints” of mature lymphocytes and other types of cell. Our results suggest either that lymphocytes of this case of CLL have T-cell differentiation markers with respect to nonspecific esterase isoenzymes, or more broadly, that peripheral T lymphocytes and B lymphocytes have similar esterase isoenzymes.

NOTE ADDED IN PROOF

Since completion of this study (Case 1 is described above) we have examined 4 additional cases of CLL (Cases 2–5), all of which tested as the typical B-cell type by standard marker tests (Table 2). All demonstrated the fine, scattered granular pattern of nonspecific esterase cytochemical reactivity in many or most lymphocytes. All showed partial fluoride resistance of...
this cytochemical reactivity, although the degree of resistance varied rather widely (Table 2).

Lymphocytes from 2 of these cases were purified from peripheral EDTA bloods (Cases 2 and 3). Specific activity of extractable nonspecific esterase was in the range of lymphocytes shown in Table 1 (mU aNB and aNA esterase respectively per 10^7 cells: Case 2, 4.3, 4.1; Case 3, 5.8, 5.8). Isoelectric focusing zymograms of aNA esterase from Cases 2 and 3 showed the usual lymphocyte pattern illustrated above (Fig. 5), as did zymograms of aNB esterase. These results suggest that the nonspecific esterase patterns described herein are general patterns for CLL B lymphocytes as well as normal T lymphocytes.

ACKNOWLEDGMENT

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