The Kinetic Properties of the Ecto-ATPase of Human Peripheral Blood Lymphocytes and of Chronic Lymphatic Leukemia Cells

By Helmut R. Gutmann, Yeh Mei Chow, Robert L. Vessella, Beth Schuetzle, and Manuel E. Kaplan

This study examines whether the activity of the Mg2+-dependent ecto-ATPase of the surface membrane of the human lymphocyte is changed in chronic lymphocytic B-cell leukemia (CLL-B) and may be an indicator of malignant transformation. The ecto-ATPase activities of preparations consisting predominantly of T or B cells were compared to each other and to the ecto-ATPase of the CLL peripheral blood lymphocytes (PBL). The specific activities and kinetic constants of the ecto-ATPase of the cell preparations were determined with [γ-32P] adenosine triphosphate (ATP) as substrate. B-enriched lymphocytes had nearly fourfold greater specific activity and apparent Vₐₘₙ values than T-enriched lymphocytes, while the Kₐ values of both cell types showed no significant difference. The specific activities and kinetic constants of the ecto-ATPase of the CLL PBL were significantly higher than the corresponding values of PBL or of B-enriched lymphocytes. Judging from the kinetic constants the ecto-ATPase of the CLL-B lymphocyte appears to be an enzyme that is distinctly different from that of the normal B cell. On the basis of the kinetic properties, the ecto-ATPase of the B cell appears to be identical with that of the T cell. The differences in the maximal velocities of the hydrolysis of ATP by B and T cells are likely due to a greater number of enzymatic sites on the B cell.

Evidence has accumulated that malignant lymphoid diseases are accompanied by alterations of the activities of ecto-nucleotide phosphohydrolases associated with the outer surface of the cell membrane. For example, the activity of the ecto-AMPase (5'-nucleotidase) was markedly decreased in human leukemic lymphocytes. It has recently been shown that the ecto-ATPase activity of the human leukocyte is significantly increased in human lymphocytes transformed by Epstein-Barr virus. Other reports indicate that the activity of the ecto-ATPase of transformed cell lines of several mammalian species is drastically altered. These reports prompted us to investigate whether the kinetic properties of this ectoenzyme are changed in chronic lymphatic leukemia and whether ecto-ATPase activity may be used as a biochemical marker of chronic lymphatic leukemia of B-cell origin (CLL-B). For this purpose, the ecto-ATPase activities of normal cell preparations enriched in T and B lymphocytes were compared to each other and to the ecto-ATPase activity of the CLL-B cell.

Materials and Methods

Subjects of Study

Peripheral blood samples were obtained from healthy volunteers randomly selected from laboratory personnel of the VAMC, Minneapolis, MN, and from CLL patients cared for by the Hematology Division, VAMC, Minneapolis, MN.

Preparation of Cells

Peripheral Blood Lymphocytes (PBL)

PBL were prepared essentially by the published procedure (method 2). The isolated PBL were washed twice with Hanks’ balanced salt solution (HBSS) and resuspended in HBSS. When PBL were depleted of T cells, HBSS was supplemented with fetal bovine serum (FBS) to give a 10% FBS concentration. The concentration of PBL in suspension (HBSS or HBSS-10% FBS) was 4 x 10⁶ cells/ml.

Depletion of B Lymphocytes

PBL were depleted of B cells by a modification of the “panning” method. Briefly, a polystyrene Petri dish (100 mm x 15 mm) was coated with Avidin DX (Vector Laboratories, Inc., Burlingame, CA) by incubating the dish with 3 ml of a 50 mM NaHCO₃ buffer (pH 8.5), containing 0.6 mg Avidin DX, for 30 min at room temperature. The solution was decanted and the plate was washed twice with buffer (2 ml) containing 0.85% NaCl. PBL were pelleted and resuspended in a solution of biotinylated goat anti-human polyvalent immunoglobulin (lg) (TAGO, Burlingame, CA), diluted sixfold with HBSS, to give a concentration of 20 x 10⁶ cells/ml. After incubation for 40 min at 4°C, the cells were washed twice and resuspended in “panning” buffer (10 x 10⁶ cells/ml). Aliquots of the cell suspension (3 ml) were poured onto one Avidin-coated plate (30 x 10⁶ cells/plate) and incubated on a level surface for 1 hr at 4°C. The nonadherent, T-enriched cells were decanted, washed twice with HBSS, and assayed for enzyme activity as described below. Only cell preparations with >95% viability (trypan blue exclusion) were used for enzymatic assays. The percentage of T cells in 10 different T-enriched cell preparations, determined as described below, was 92.8 ± 1.8 (mean ± SD).

Depletion of T Lymphocytes

T-cell rosettes were prepared by the large-scale modification of the published procedure. The rosetted cells were decanted, washed twice with HBSS, and assayed for enzyme activity as described below. Only B-enriched cells with >95% viability were used in enzyme

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studies. The percentage of surface immunoglobulin (SIg) positive cells in 14 different B-enriched lymphocyte preparations was 81.5 ± 5.5.

**CLL PBL**

CLL PBL from leukemic donors were obtained and treated prior to the enzymatic assay as indicated above. The viability of these cells was >95% in all cases. The percentage of SIg-positive cells in 12 different preparations was 86.5 ± 13.8.

**Determination of the Purity of Separated Lymphocyte Subfractions**

The purity of the B- and T-enriched lymphocyte preparations was assessed by rosetting with sheep red blood cells (SRBC) pretreated with S-(2-aminoethyl)-isothiouronium bromide (AET, obtained from Aldrich Chemical Co., Milwaukee, WI) and by immunofluorescence utilizing biotinylated goat anti-human polyclonal Ig and fluoresceinated Avidin (Vector Laboratories, Inc., Burlingame, CA).

**Enzyme Studies**

**Incubation Conditions**

Prior to incubation, PBL, T-enriched and B-enriched lymphocytes, and CLL PBL were washed twice with incubation medium. The medium consisted of Tris-HAc buffer (30 mM, pH 7.4), NaCl (130 mM), KCl (10 mM), and MgCl2 (2 mM). A medium of similar ionic composition and strength was used in a previous investigation of the ecto-ATPase activity of human leukocytes. The mono- and divalent cations were found to be essential for optimal activity of the ecto-ATPase of PBL. Only Na+ and K+ ions were required for optimal activity of the ecto-AMPase. Since Mg2+ had no effect on ecto-AMPase activity, the above medium was used for the determination of the activity of both ecto-enzymes. Incubations were carried out in a rotary shaker at 37°C for 20 min, during which time cell viability remained >95%. To insure intactness of the cell membrane, longer incubation times were avoided. The activity of both ecto-enzymes was linear for at least 20 min.

For the determination of ecto-AMPase activity of PBL or CLL PBL, the standard assay mixture (0.5 ml) consisted of 5–10 x 10^6 cells and AMP (0.5 mM). For the determination of ecto-ATPase activity of PBL, T- and B-enriched lymphocytes, and CLL PBL, the numbers of cells used were kept within the linear range of plots relating cell number of each cell population to ecto-ATPase activity (Fig. 1). The concentration of [γ-32P]ATP in the incubation mixtures (0.5 ml) ranged from 0.025 to 0.5 mM, and the radioactivity/incubation mixture was 80,000–100,000 cpm. After 20-min incubation, proteins were precipitated with cold 11% TCA and removed by centrifugation. Duplicate or triplicate blanks containing labeled substrate without cells were run concurrently. When the ecto-ATPase activity of B-enriched lymphocytes was determined, blanks containing labeled substrate and AET-treated SRBC equal to the number of SRBC in the lymphocyte preparation were also assayed.

**Calculation of Kinetic Constants of Ecto-ATPase**

Values of apparent K_m and apparent V_max were calculated on a Hewlett Packard 9830 A calculator with a program using the weighted least-squares method.** Statistical analysis of the data (means ± SD, significance of differences of means) was performed with computerized programs of the standard procedures.

**Source of Nucleotide Phosphates**

[γ-32P]ATP, as the Tris(hydroxymethyl)methane salt prepared according to the published procedure, was generously supplied by Dr. K. Ahmed and Mr. A. Davis, VAMC, Minneapolis, MN. The sodium salts of AMP and ATP were purchased from Sigma Chemical Company, St. Louis, MO.

**RESULTS**

In initial experiments (Table 1), the ecto-ATPase activities of PBL and of CLL PBL were compared at 0.5 mM [γ-32P]ATP. The average specific activity of

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**Fig. 1.** Formation of 32P, from [γ-32P]ATP as a function of the number of cells. The isolation and fractionation of lymphocytes, the incubation conditions, and the method for the assay of 32P are described under Materials and Methods. The substrate concentration was 0.5 mM. The following cell types were used in the assays: curve I, PBL; curve II, B-enriched lymphocytes; curve III, T-enriched lymphocytes; curve IV, CLL PBL cells. Each point on the respective curves is the average of duplicate or triplicate determinations.
ECTO-ATPase OF LYMPHOCYTES

Table 1. Specific Activities of the Ecto-ATPase of PBL and CLL

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Nanomole 32P</th>
<th>Apparent Velocity (Vmax) Formed/10^8 Cells/20 min</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>2.2-10.5</td>
<td>6.0 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>CLL PBL†</td>
<td>8.6-41.1</td>
<td>23.6 ± 8.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*The samples for the assay of PBL were obtained from male (5) and female (9) donors. The difference between the formation of 32P, by male and female donors was statistically not significant (0.50 < p > 0.40).
†Samples obtained from 18 male donors.

the ecto-ATPase of CLL PBL was nearly fourfold greater than that of the ecto-ATPase of PBL. There was no correlation between the enzyme activity and the age of the patient.

Two possibilities were considered to explain the marked increase of the ecto-ATPase of CLL PBL. First, as a result of the transformational process, the ecto-ATPase of the CLL B lymphocytes might be an enzyme with kinetic properties distinctly different from that of the normal B lymphocyte. On the other hand, the ecto-ATPase of the CLL PBL might be the same enzyme as that associated with the normal B cell. If this were the case, the observed increase in the activity of CLL PBL would be accounted for by the preponderance, in CLL PBL, of B cells with high ecto-ATPase activity. The marked differences in the activities of the PBL and CLL cells would imply that the ecto-ATPase of the T cell, which comprises ~80% of PBL in normal individuals, is significantly less active than the ecto-ATPase of the B cell. To decide between these possibilities, the ecto-ATPase activity of lymphocyte preparations enriched in B or T cells was examined.

Determination of the kinetic constants (apparent Km and apparent Vmax) of the hydrolysis of [γ-32P]ATP by the ecto-ATPase of T- and B-enriched lymphocytes (Table 2) showed that the average maximal velocity of B-enriched cells was approximately threefold greater than that of T-enriched lymphocytes (p < 0.001). In contrast, there was no significant difference between the apparent Km of the ecto-ATPase of T-enriched and of B-enriched cells. The values for Km of the ecto-ATPase of T- and B-enriched cells were of a similar magnitude as those reported for human granulocytes (0.05 mM) and for unfractionated human lymphocytes (0.06 mM). The equal values for the apparent Km of the ecto-ATPase of T- and B-enriched cells suggest that the ecto-ATPases of the two subpopulations of PBL are identical enzymes. The differences in the average maximal velocities of T- and B-enriched cells may be due to a greater concentration of the same ecto-ATPase on the surface membrane of the B cell.

Since lymphocyte preparations enriched in B cells contained, on the average, 19% T cells, the question arose whether the kinetic constants of the ecto-ATPase of B-enriched cells (Table 2) represented accurate values that could be used without correction for a comparison with the kinetic constants of CLL PBL. To settle this point, paired PBL preparations of 6 donors were depleted of B and T cells, and the ecto-ATPase activities of the T- and B-enriched preparations were determined separately at 5 different substrate concentrations. The amounts of 32P, formed by the T cells in the B-enriched preparations were subtracted from the quantities of 32P, formed by the B-enriched cells. The corrected values, which represent the amounts of 32P, released by the ecto-ATPase of the B cells in the B-enriched preparation, were used to calculate the specific activities and kinetic constants of the B lymphocytes. Comparison of the kinetic constants of the ecto-ATPase of B-enriched cells, without and with correction for the ecto-ATPase of T cells in B-enriched preparations containing 9%-27% T cells, indicated that correction did not change the values of Km or Vmax significantly (Table 3). As long as contamination by T cells did not exceed approximately 25% of the B-enriched lymphocyte population, uncorrected kinetic constants appeared to be a valid measure of the ecto-ATPase activity of the B cell and were used to compare the ecto-ATPase activity of the B cell with that of CLL PBL.

Measurements of the ecto-ATPase activity of CLL PBL and of B-enriched cell preparations containing at least 75% SIg-positive cells indicated that the mean values for apparent Km and Vmax of CLL PBL were

Table 2. Kinetic Constants of the Ecto-ATPase of T-Enriched and B-Enriched Lymphocytes*

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Apparent Km</th>
<th>Apparent Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>Mean ± SD</td>
<td>p</td>
</tr>
<tr>
<td>T-enriched lymphocytes†</td>
<td>0.03-0.13</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>B-enriched lymphocytes‡</td>
<td>0.03-0.18</td>
<td>0.10 ± 0.04</td>
</tr>
</tbody>
</table>

*The kinetic constants were calculated from the specific activities of duplicate samples determined at 5 different substrate concentrations as described in Materials and Methods.
†Samples were obtained from 7 male donors.
‡Samples were obtained from 10 male and 4 female donors. There was no significant difference between the apparent Km and apparent Vmax of the samples (p < 0.05).
ATPase activities of the B-enriched cells and of a mixture consisting of B-enriched cells and of T-cell suspensions were determined at 3 substrate concentrations (column 4, Table 5).

To resolve this problem, the ecto-ATPase activity of cell suspensions consisting of a mixture of B-enriched cells and of CLL PBL was determined at 3 substrate concentrations (column 4, Table 5). Simultaneously, the ecto-AMPase activity of the cell mixture was only 15% greater than the activity calculated from the individual activities of the 2 cell types.

The question was raised whether the increased activity of the ecto-ATPase of PBL and of the ecto-ATPase activity of CLL PBL is attributable to an activator associated with the CLL B lymphocyte. It has been reported that the ecto-ATPase activity of CLL PBL was the same as that obtained by assay of the cell mixture. At 2.0 and 0.5 mM [γ-32P]ATP, the rate of substrate hydrolysis calculated from the ecto-ATPase activities of B-enriched cells and from CLL PBL was the same as that obtained by assay of the cell mixture. At 0.05 mM [γ-32P]ATP, the ecto-ATPase activity of the cell mixture was only 15% greater than the activity calculated from the individual activities of the 2 cell types. These data do not support the view that an activator associated with the CLL B lymphocyte accounts for the increased ecto-ATPase activity of CLL PBL. Similarly, it seems unlikely that the lower activity of the ecto-ATPase of the normal B cell is due to an inhibitor present in the B cell.

Although our results indicated a marked increase of the average activity of the ecto-ATPase of CLL PBL, the usefulness of the assay for the diagnosis of CLL-B leukemia is open to question because of the overlap of the values of the ecto-ATPase activity of normal and leukemic lymphocytes. It has been reported that the ecto-AMPase activity of CLL PBL is markedly lower than that of PBL and of T- or B-enriched cell preparations.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Percent T Cells in B-Enriched Cell Preparations</th>
<th>Apparent $K_m$ of B-Enriched Cells</th>
<th>Apparent $V_{\text{max}}$ of B-Enriched Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uncorrected Corrected for T-Cell Activity†</td>
<td>Uncorrected Corrected for T-Cell Activity†</td>
</tr>
<tr>
<td>1</td>
<td>27</td>
<td>0.11 0.12</td>
<td>26.4 33.2</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0.07 0.07</td>
<td>16.7 18.0</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>0.04 0.04</td>
<td>9.7 10.6</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>0.14 0.14</td>
<td>28.0 30.1</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>0.06 0.06</td>
<td>9.7 10.2</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>0.03 0.04</td>
<td>15.8 17.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Range Mean ± SD</th>
<th>Range Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08 ± 0.04‡</td>
<td>17.7 ± 7.9‡</td>
</tr>
<tr>
<td></td>
<td>20.9 ± 9.7‡</td>
</tr>
</tbody>
</table>

*The values listed for each donor are the averages of duplicate determinations. Kinetic constants were calculated from the specific activities determined at 5 different substrate concentrations as described in text.
†Corrections were made by determining separately the specific activities of T-enriched lymphocytes as described in the text. The nanomoles $^{32}$P, formed by the T cells in the B-enriched cell preparation were calculated and subtracted from the total nanomoles $^{32}$P, formed by the B-enriched cells. This value, divided by the number of B cells in the B-enriched preparation, is the specific activity of the B-enriched cells corrected for T-cell activity.
‡Means ± SD
§The values of $V_{\text{max}}$ of B-enriched cells (A) uncorrected and (B) with correction for T-cell activity are not significantly different by paired t test statistics ($p > 0.05$).

Table 4. Kinetic Constants of the Ecto-ATPase of B-Enriched Lymphocytes and of CLL PBL*

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Apparent $K_m$</th>
<th>Apparent $V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range Mean ± SD</td>
<td>Range Mean ± SD</td>
</tr>
<tr>
<td>B-enriched lymphocytes†</td>
<td>0.03-0.18 0.09 ± 0.05</td>
<td>0.7-28.2 19.1 ± 6.9</td>
</tr>
<tr>
<td>CLL PBL‡</td>
<td>0.08-0.52 0.31 ± 0.16 &lt;0.001</td>
<td>9.3-66.9 37.8 ± 17.5 &lt;0.005</td>
</tr>
</tbody>
</table>

*The kinetic constants were calculated from the specific activities of duplicate samples determined at 5 different substrate concentrations as described under Materials and Methods.
†Samples were obtained from 10 male donors.
‡Samples were obtained from 12 male donors.
whether the change in activity of the ecto-AMPase is consistent with that found for ecto-ATPase, and (2) which of the two ecto-enzymes would be the more reliable indicator of CLL-B leukemia. The assays (Table 6) indicated that the average activity of the ecto-AMPase of CLL PBL was decreased consistently by >90% in comparison to that of PBL. Furthermore, there was no overlap in the values of normal and leukemic cells. Diminished ecto-AMPase activity appears to be reliable evidence for CLL-B leukemia. The observation that malignant transformation of the normal lymphocyte to the CLL cell has opposite effects on the hydrolysis of AMP and ATP suggests that ecto-AMPase and ecto-ATPase are separate enzymes of the lymphocyte membrane.

**DISCUSSION**

The present study provides the first evidence, by a quantitative method, for a significant elevation of the activity of the ecto-ATPase of the CLL B cell relative to that of the ecto-ATPase of the normal B cell. Prior to this study, a Mg++-dependent ATPase had been suggested as a marker of malignant transformation of the B cell to the CLL B cell. This suggestion was based on the observation that a strong ATPase reaction could be demonstrated histochemically in the plasma membrane of the B-lymphoma cell. Although the histochemical method is not specific for ATPase, the requirement for Mg++ suggests that the reaction noted by these investigators may be attributed to increased ecto-ATPase activity. Two mechanisms may be considered as underlying the increased activity of the ecto-ATPase of the CLL B cell. First, consistent with current hematologic concepts, a B-cell clone may proliferate and replace the normal lymphoid cell population. This clone may possess an ecto-ATPase with high K_m and V_max. It is unknown why this particular clone would be selected for proliferation in the transformational process. However, this possibility cannot be verified at present because currently available biochemical techniques are not sufficiently sensitive to measure the ecto-ATPase activities of single lymphoid cells or clones. As an alternative hypothesis, one may postulate that the increased ecto-ATPase activity of the CLL B lymphocyte is related to similar changes seen in other mammalian cells undergoing malignant transformation. For example, the Mg++-dependent ecto-ATPase activity of cultured rat hepatocytes increased appreciably when the cells were transformed by exposure to chemical carcinogens. A correlation between the oncogenic potential of cultured rat liver epithelial cells, complexities of morphological changes of the cell membrane, and the rate of hydrolysis of [γ-32P]ATP by membrane ATPase has also been noted. We suggest that the altered kinetic properties of the ecto-ATPase of the CLL B cell reflect alterations of the cell membrane coincident with the process of malignant transformation and that the increase in ecto-ATPase activity may be a measure of the change of the membrane structure. In this connection, it should be mentioned that one instance of CLL-B leukemia was observed in which the kinetic constants were found repeatedly to be unusually high (K_m > 0.85 mMg, V_max > 170 n mole 32P formed/10^6 cells/20 min). These values exceeded the average K_m or V_max listed in Table 4 by 3 and 4 standard deviations, respectively, and were not used in calculating the data of Table 4, in order to avoid distortion of the calculated mean values. This particular assay indicates that very high activities of the ecto-ATPase of CLL PBL, which we interpret to be due to extensive membrane alteration of the CLL B cell, may occasionally be seen.

The present investigation shows that the average maximal velocity of the ecto-ATPase of the normal B lymphocyte is approximately 3 times greater than the corresponding value of the T cell. It is of interest that
differences of similar magnitude have been observed for the ecto-AMPase activities of B and T cells from normal subjects. It would appear that the nucleotide phosphohydrolases associated with the surface membrane of the T lymphocyte are generally less active than the corresponding ecto-enzymes of the B cell. Higher ATPase activity of the B lymphocyte compared to that of the T cell has been reported. These studies were performed on homogenates of B- and T-enriched lymphocytes with the use of unlabeled ATP. The released under these conditions may have come from the sequential hydrolysis of ATP, ADP, and AMP, since human peripheral blood lymphocytes and polymorphonuclear leukocytes contain, in addition to ecto- and endo-ATPases, an ADPase partially located at the outer surface of the cell membrane as well as an AMPase associated with the membrane of the human leukocyte or lymphocyte. The use of intact lymphocytes and of ATP labeled terminally with 32P leaves no doubt that the hydrolysis of ATP in the present study may be attributable exclusively to the action of an ecto-ATPase.

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