Studies on the Lymphocyte 5'-Nucleotidase in Chronic Lymphocytic Leukemia, Infectious Mononucleosis, Normal Subpopulations, and Phytohemagglutinin-stimulated Cells

F. Quagliata, D. Faig, M. Conklyn, and R. Silber
Department of Medicine, New York University Medical Center, New York, New York 10016

SUMMARY

5'-Nucleotidase (5'N) activity, which is present in lymphocytes isolated from the blood of normal subjects, is markedly diminished or not detectable in most patients with chronic lymphocytic leukemia (CLL). The relation to lymphocyte subpopulations and the effect of mitogenic stimulation and disease states on 5'N were investigated. The reduced activity in CLL does not stem from the increase in the percentage of B-lymphocytes found in this disorder. Stimulation of normal or 5'N-positive CLL lymphocytes with phytohemagglutinin does not lead to a decrease in activity comparable to that found in 5'N-deficient CLL cells. In contrast to what was observed with 5'N, the level of another plasma membrane marker enzyme, diphosphopyridine nucleotide diaphorase, is normal in CLL. In lymphocytes from 103 patients with a wide variety of clinical disorders, normal levels were found in all cases except during the early phase of infectious mononucleosis, when a marked decrease occurred. In certain patients with CLL the decreased enzyme level has remained constant over more than 24 months of observation; the decreased activity in infectious mononucleosis, on the contrary, a very transient finding, with a gradual return to normal levels. The lymphoblasts isolated from the blood in 2 out of 5 patients with acute myeloblastic leukemia studied had markedly decreased 5'N activity. It appears that lymphocyte 5' activity may be useful in the biochemical characterization of subgroups of patients with lymphoid leukemias.

INTRODUCTION

Recent observations from 2 laboratories indicate that 5'N activity is markedly decreased or not detectable in the lymphocytes of some but not all patients with CLL (10, 12). These studies raise several questions. (a) Since there is an increase in the percentage of B-cells in CLL (14, 16), could the absence of 5'N activity in the lymphocytes of some patients be explained on the basis of an altered B:T cell ratio? (b) Is the lack of this activity relatively specific for CLL or is the level also decreased in other clinical conditions affecting lymphocytes? (c) What is the relationship of 5'N to lymphocyte proliferation activity? The present paper addresses itself to these questions.

MATERIALS AND METHODS

Thymidine-2,14C (specific activity, 55.7 mCi/mmmole) and AM32P (specific activity, 7.0 Ci/mmmole) were purchased from New England Nuclear, Boston, Mass. The AM32P was purified by paper chromatography in isobutyric acid:ammonia:water (66:1:33) to remove 32P and was diluted to a specific activity of approximately 10,000 cpm/nmole. Neuraminidase Type VI (Vibrio cholerae) was obtained from Sigma Chemical Co., St. Louis, Mo. Trypsin (5 times crystallized) and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp., Freehold, N. J. Lymphocytes were purified from blood as previously described (9). The enriched preparation, which contained over 95% lymphocytes, was suspended in 0.05 M Tris-maleate:5% sucrose buffer, pH 7.4. Whereas 5'N activity had been previously measured by the colorimetric estimation of Pi, a more sensitive and simpler assay in which the reaction rate is determined from the generation of 32Pi, was used. The assay was performed in a total volume of 0.1 ml and contained the following: Tris-maleate buffer, pH 7.4, 250 nmoles; /3-glycerophosphate, 2 jimoles; and AM32P, 40 nmoles. The reaction was started by the addition of the substrate (5'-AM32P), but not the 32Pi product, was adsorbed to Norit A. The assay was performed in a total volume of 0.1 ml and contained the following: Tris-maleate buffer, pH 7.4, 250 nmoles; sucrose, 7.3 μmoles; sodium-potassium tartrate, 1 μmole; β-glycerophosphate, 2 μmole; and AM32P, 40 nmoles. The reaction was started by the addition of the substrate. In most experiments, between 0.4 and 2 × 106 lymphocytes were added to the assay mixture. At the end of the incubation period, which was usually 30 min, 0.2 ml of 10% TCA and 100 μl of 20% Norit A were added, and the samples were left on ice for 10 min. The samples were then centrifuged at 1900 X g for 5 min. Ten ml of Bray's solution were added to a 0.1-ml aliquot of the supernatant fluid, and radioactivity was determined in a Packard liquid scintillation counter. TCA was added to the blank prior to incubation. Duplicate determinations agreed within ± 15%. The results obtained with this assay were identical to those in which the liberation of Pi had been determined colorimetrically. The reaction rate was generally linear for up to 2 hr of incubation. Similar specific activities

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were obtained when intact or disrupted lymphocytes were used as the source of enzyme. Protein concentration was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard. 5'N specific activity is expressed as µmoles/mg/hr or µmoles/10^8 lymphocytes/hr. The 5'N values in the lymphocytes from 3 normal subjects and 3 patients with CLL studied over a 30-month period did not fluctuate beyond ±25%. The age and sex of the lymphocyte donor were found to have no effect on the level of activity. DPNH diaphorase activity was determined according to the method of Wallach et al. (18); specific activity is expressed as µmoles/min/mg. The clinical criteria for the diagnosis of CLL were as previously reported (10).

Determination of Percentage of B-Cells and T-Cells. The percentage of lymphocytes bearing receptor for complement (B-cells) was determined by a minor modification (19) of the method of Bianco et al. (1). Selective depletion of B- and T-cells was achieved as described by Broome et al. (3). The remaining cells were then washed 3 times in Roswell Park Memorial Institute 1640 Medium and processed as described. T-lymphocytes, i.e., the percentage of cells having receptors for sheep erythrocytes were measured by the procedure of Lay et al. (8) as modified by Weiner et al. (19). Only lymphocytes with 3 or more attached erythrocytes were counted as rosettes in the determination of T- and B-cells.

PHA Stimulation Studies. Purified lymphocytes were cultured as previously described (17) with a substitution of Roswell Park Memorial Institute Medium 1640 with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer for Minimum Essential Medium as a growth medium. Reagent grade PHA Lot No. HA15 (Wellcome Research Laboratories, Beckenham, England) was added in amounts of 100, 10, or 1 µg/4 ml of culture medium. For each experimental point shown, culture tubes were set up in sextuplicate. To 3 of these, 0.1 µCi of thymidine-2-14C was added at the time interval specified under “Results,” and incorporation of radioactivity into TCA-precipitable material was determined. The other 3 samples were used for 5'N assays.

RESULTS

5'N in B- and T-Lymphocytes

The relationship of 5'N activity to the percentage of B- and T-cells was evaluated in the following 3 types of experiments.

1. Studies on CLL Lymphocytes. The 5'N level in lymphocytes from individual patients with CLL is plotted against the percentage of B- or T-cells in Chart 1. Among 13 patients, a normal 5'N level was found in 4 (Patients 3, 4, 7, and 10). The lack of a relationship between 5'N activity and T-cells is suggested by the finding that the percentage of T-cells in one of these cases (Patient 7) was at the lower limit of the normal range, while the percentage was markedly decreased in the other 3 patients. Further evidence against a dependency of the activity on the percentage of T-cells is shown by the observation that Patients 3, 4, 9, 10, 11, and 12, who show a wide scatter of 5'N activity, had a comparable percentage of T-cells. Similarly, the independence of cell type and 5'N level is also shown in Chart 1, Column A. All the patients studied had an elevated percentage of B-cells. Very low (or absent) 5'N was found in patients with minimal increase in the percentage of B-cells (Patients 6, 8, and 13) as well as in other (Patients 1 and 9) whose lymphocytes consisted almost entirely of B-cells. Among the 4 patients whose 5'N activity was normal, the percentage of B-cells varied from minimally increased in Patient 7 (20%) to a marked elevation in Patients 3 and 4, who had over 70% B-cells. The 5'N activity therefore appears to be independent of the percentage of either cell type.

2. Depletion of B- and/or T-Cells. If the 5'N activity were localized preferentially to either B- or T-cells, a decrease in activity after depletion of one of these cell types would be expected. The following experiments were therefore performed. Lymphocyte populations containing T- and B-cells were treated as described under “Materials and Methods” for the removal of either cell type. Since the incubation and washings

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![Chart 1](image-url)
might result in some decrease in activity, control cells were subjected to all manipulations except for the omission of trypsinized sheep erythrocytes coated with amboceptor and complement in the B-depletion experiments and of sheep erythrocytes in the T-depletion experiments to prevent the binding and therefore the removal of these cells. These “sham-depleted cells” served as control for the effect of the procedure alone on enzyme activity. In 1 experiment, lymphocytes containing 40% T-cells with a 5’N activity of 0.18 after T-cell depletion showed a reduction in T-cells to 1.7%, with an insignificant reduction of enzyme activity to 0.13 μmole/hr/mg. In a 2nd experiment, lymphocytes with 3.5% B-cells and a 5’N activity of 0.49 contained 0.1% B-cells and a 5’ level of 0.54 μmole/hr/mg after depletion. Each of these experiments was repeated with 3 other normal donors. It was also performed in a patient with CLL whose 5’N activity before selective depletion was 1.3 μmole/hr/mg. After removal of B-cells, the activity was 1.8 μmoles/hr/mg, and following depletion of T-cells, it was 1.7 μmoles/hr/mg. The specific activity after depletion of T- or B-cells (or T- and B-cells) was always comparable to that of the “sham-depleted” control cells. These results indicate that, since comparable 5’N activity was found after the selective removal of either lymphocyte population in normal subjects or patients with CLL, the enzyme is not contributed exclusively by either cell type.

3. 5’N in Blood, Tonsil, or Thymus Lymphocytes. The results of the 3rd type of experiment performed in order to evaluate the relationship of 5’N to T- and B-cells are shown in Table 1, where the relative percentages of these cells in blood, tonsil, and thymus lymphocyte populations are given. These suggest that the low 5’N in CLL cannot be ascribed to the decrease of T-cells in this disorder, since thymocytes consisting almost entirely of T-cells have a relatively low enzyme level rather than the high activity that might be expected were the enzyme preferentially localized to T-cells. Evidence that the increase in B-cells in CLL is not responsible for the lack of 5’N is also provided by the data on tonsil lymphocytes. The high percentage of B-cells in this tissue is comparable to that found in most CLL patients, but 5’N activity in tonsil lymphocytes is similar to that of normal blood lymphocytes; despite the high percentage of B-cells, there is no decrease in 5’N.

### Table 1

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>5’N specific activity (μmole/hr/mg)</th>
<th>T-cells (%)</th>
<th>B-cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus (3)</td>
<td>0.17</td>
<td>97</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil (3)</td>
<td>0.7</td>
<td>45</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>33</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.21</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Blood (7)</td>
<td>0.39</td>
<td>42.6</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>(0.18—0.6)</td>
<td>(30—53)</td>
<td>(3.0—13)</td>
</tr>
</tbody>
</table>

| a Mean values and range.  
| b Numbers in parentheses, number of cases.  

### Lymphocyte 5’N in Various Disorders

5’N levels were determined in lymphocytes from patients with a wide variety of clinical disorders. The results are shown in Table 2. In cardiopulmonary disorders, the specific activity of 5’N was similar to that of normal lymphocytes. Lymphocytes from patients with infections, which included bacterial infections and viral infections (mumps, hepatitis, and nonbacterial respiratory infections), had levels within the normal range. 5’N activity was also within normal limits in the lymphocytes of 3 patients with leprosy, a disorder in which the cellular immune response is depressed; PHA reactivity is diminished, and a higher than normal percentage of B-cells is found (6, 7). Patients with dermatological disorders, which at times are associated with an increase in T-lymphocytes (4, 20), also had normal levels of 5’N. Finally, normal levels were also found in lymphocytes isolated from the blood of patients with acute lymphocytic leukemia (21), B-lymphocytic leukemia (19), and lymphoma (8, 22, 23, 24). In lymphoma, the 5’N activity was 0.25 (0.09—0.49) pmoles/hr/mg. Clinical category | No. | 5’N specific activity (μmole/hr/mg)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiopulmonary</td>
<td>21</td>
<td>0.35 (0.09—0.70)</td>
</tr>
<tr>
<td>Metabolic</td>
<td>5</td>
<td>0.30 (0.10—0.49)</td>
</tr>
<tr>
<td>Infections (viral and bacterial)</td>
<td>19</td>
<td>0.27 (0.10—0.60)</td>
</tr>
<tr>
<td>Dermatological</td>
<td>7</td>
<td>0.25 (0.09—0.49)</td>
</tr>
<tr>
<td>Miscellaneous hematological disorders</td>
<td>6</td>
<td>0.25 (0.11—0.70)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>15</td>
<td>0.30 (0.10—0.70)</td>
</tr>
<tr>
<td>Neoplastic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma and sarcoma</td>
<td>12</td>
<td>0.34 (0.06—0.52)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>6</td>
<td>0.33 (0.12—0.61)</td>
</tr>
<tr>
<td>Acute lymphocytic leukemia</td>
<td>2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.03—0.09</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.10—1.70</td>
</tr>
<tr>
<td>IMb</td>
<td>2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.03—0.09</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.10—0.29</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>0.40 (0.10—0.90)</td>
</tr>
</tbody>
</table>

a Mean values and range. The differences between CLL, IM, and normal subjects (or patients with other disorders) are also found when the specific activity is expressed as μmoles/hr/10^7 cells.  

b Initial values only are given in this table.
with carcinomas, sarcomas, and lymphomas as well as from subjects who were anemic from blood loss or hemolysis.

Also shown in this table are the results of S'N assays in 27 patients with CLL. These data, which include the 9 patients previously reported (10), confirm our earlier finding that heterogeneity exists for the activity of this enzyme. The patients have been arbitrarily divided into 3 groups: 1 in which the specific activity is markedly decreased or absent (<0.03), another with subnormal levels (0.03 to 0.09), and a 3rd group of patients whose enzyme levels were comparable to those of normal lymphocytes. One of the patients in the latter group (Chart 1, Case 4) actually had a higher S'N specific activity than any normal subject tested. The number of cases studied is insufficient to decide whether these are true subgroups or a spectrum ranging from undetectable to normal or even supranormal activity levels.

The results in the 5 patients with acute lymphocytic leukemia studied suggest that heterogeneity may also exist in the lymphoblast S'N activity, since the activity was low in 2 patients and normal in the others.

An unexpected observation was made on patients with IM. The activity in the lymphocytes from 5 of 7 patients with this disorder was markedly decreased or undetectable when assayed at the time the diagnosis was made. A striking difference was found, however, in the activity pattern of this enzyme in CLL and IM. Whereas, as has been previously reported (and also noted in the present study), patients with CLL lacking S'N did not show a return of the activity when their lymphocytes were assayed repeatedly over periods of more than 22 months, the decrease of S'N in IM was an early and transient phenomenon. As shown in Chart 2, the activity, markedly reduced when first tested, started to increase as early as within 2 days, generally reaching normal levels within 2 weeks. The S'N level begins to increase at a time when atypical lymphocytes, abnormal liver function tests, and an elevated heterophile reaction are still present.

To evaluate whether the diminished S'N in CLL lymphocytes was associated with a decrease in the activity of other membrane enzymes, the activity of DPNH diaphorase, another well-established plasma membrane marker (18), was determined in lymphocytes from 10 patients with CLL and 10 normal subjects. The specific activity in the lymphocytes of patients was 0.13 (range, 0.09 to 0.23), which is not significantly different from the normal average of 0.10 (range, 0.05 to 0.17).

**PHA Stimulation Studies**

Since the decrease in S'N in CLL and IM might be a reflection of altered mitotic status, it seemed appropriate to determine the relationship of mitogenic stimulation to S'N level. Lymphocytes from 4 normal subjects and 4 patients with CLL were incubated with concentrations of PHA adjusted to give maximal stimulatory effect, and the relationship of enzyme level to PHA response, as measured by thymidine-2,14C incorporation, was determined. The results of these experiments, shown in Table 3, reveal the following: (a) the S'N activity in lymphocytes from normal subjects remained unchanged after 3 days of culture in the presence of PHA and showed a moderate (25 to 50%) decrease after 5 days. The latter probably does not reflect a diminution in total S'N activity per cell but may represent a decline in the specific activity of this surface enzyme due to the decreased surface per volume of the larger, stimulated cells; (b) lymphocytes from CLL Patients 2 and 8 lacking S'N before exposure to PHA did not develop detectable levels of activity after the cells were cultured in the presence of the mitogen. In CLL cells with normal S'N activity (Patients 7 and 9), a positive PHA response was associated with a moderate decrease in the specific activity of S'N as in lymphocytes of normal subjects.

PHA is considered predominantly to stimulate T-cells (5), while pokewood and streptococcal mitogens will also stimulate B-cells (5, 15). When the effect of these agents on lymphocyte S'N activity was determined, the results were similar to those observed with PHA.

The mitogenic effect of PHA as measured by the incorporation of thymidine-2,14C was evaluated in 11 patients with CLL. No difference was observed between the positive PHA responses of S'N-positive and S'N-negative cell populations.

**DISCUSSION**

The data presented above clearly indicate that the marked decrease of S'N in the lymphocytes of some patients with CLL and the presence of normal levels in cells from others is independent of the increased percentage of B-cells found in subjects with this disorder. Evidence that S'N activity is not a reflection of the B- and T-subpopulations was provided by the results from 3 types of experiments. 1, the percentage of neither B- nor T-cells in patients with CLL showed a positive correlation with S'N activity. 2, the selective depletion of B- or T-lymphocytes failed to alter the specific activity of S'N. 3, S'N activity in various lymphoid organs did not correlate with the relative number of B- and T-lymphocytes.

Studies on the lymphocytes from patients with a wide variety of clinical disorders indicate that the specific activity of S'N was not affected by many cardiopulmonary, metabolic, neoplastic, dermatological, and infectious disorders with the exception of IM, in which a marked but transient decrease in
activity was noted during the early stages of the disease. Experiments with PHA revealed that, unlike the case with lysosomal enzymes, the activity of which is stimulated by the mitogen (2), the addition of PHA to the culture medium had relatively little effect on S'N. It therefore appears that the failure to detect activity in some patients with IM and CLL is unrelated to the overall increased mitotic activity of the lymphocytes in these disease processes.

The finding of a decrease in S'N in IM indicates that this enzymatic alteration is not limited to the CLL lymphocyte. The low S'N activity apparently does not represent a generalized decrease of plasma membrane enzymes in CLL, since the level of DPNH diaphorase, an enzyme with identical subcellular localization, was similar to the levels of normal lymphocytes.

The studies reported above do not establish the cause of the decrease in S'N. Two possibilities have been considered that would explain these observations. The first is that S'N-positive and -negative populations exist in normal lymphocytes. If a mutation occurs in a S'N-positive clone, the patient will develop S'N-positive CLL, while a mutation in a S'N-negative clone would give rise to S'N-negative CLL. The transient loss of the activity in IM may be a reflection, early in the course of this disease, of the proliferation of a population of lymphocytes devoid of this enzyme or the loss of the activity in a preexisting population. The latter hypothesis would be consistent with a mechanism by which an infectious viral agent might interfere with the synthesis of this cell surface constituent. Available data do not permit a decision as to whether either of these possibilities is correct. IM, a disease caused in all probability by Epstein-Barr virus, shares a number of characteristics with malignant lymphoproliferative disorders, i.e., elevated levels of dihydrofolate reductase and the occurrence of immune hemolytic anemia and thrombocytopenia. The lack of S'N adds another biochemical similarity between this benign self-limited disorder and CLL.

Regardless of the etiology underlying the lack of S'N, the marked decrease of this activity in the lymphocytes of some patients with CLL indicates that, despite the morphological similarity, the presence of biochemical heterogeneity can be used to subdivide patients with CLL into S'N-positive and S'N-negative subjects. The possible clinical significance of these subgroups in terms of the natural history of the disease and response to therapy must be established.

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REFERENCES

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