Adenosine and Adenosine Analogues Are More Toxic to Chronic Lymphocytic Leukemia Than to Normal Lymphocytes

By S. Bajaj, J. Insel, F. Quagliata, R. Hirschhorn, and R. Silber

We compared the effect of adenosine and adenosine analogues on the phytohemagglutinin-induced proliferative response of blood lymphocytes from normal subjects and patients with chronic lymphocytic leukemia. As measured by the inhibition of thymidine or leucine incorporation, adenosine was more toxic to chronic lymphocytic leukemia (CLL) than to normal lymphocytes. This difference was not affected by the removal of adherent cells. The patients' B lymphocytes were more susceptible to adenosine toxicity than normal B lymphocytes. Similar responses were noted in T lymphocytes from both sources. Differential susceptibility was also observed with deoxyadenosine and adenosine analogues, including 5'-deoxycytidine. Urine rescue from adenosine toxicity was observed for normal and CLL lymphocytes. In the presence of uridine, there was no difference in the residual inhibition of CLL as compared to normal lymphocytes. Intact CLL lymphocytes metabolized [14C]-adenosine at a much lower rate than normal lymphocytes. While it appears that the greater toxicity of adenosine to CLL lymphocytes reflects the impaired catabolism of this nucleoside by these cells, evidence is presented that this is not the only mechanism underlying the differential susceptibility. These results may serve as the basis for further pharmacologic investigations of adenosine and adenosine deaminase inhibitors in chronic lymphocytic leukemia.

MATERIALS AND METHODS

Blood was drawn into heparinized syringes from normal donors and patients with B-cell CLL. The diagnosis of CLL was based on standard criteria, including bone marrow examination. The patients were either untreated or had not received therapy for 1 yr prior to the study. Informed consent was obtained from all patients according to the provisions of the Helsinki conference. Normal leukocyte-rich blood used for isolation of T and B cells was obtained from the New York Blood Center.

Mononuclear cells were isolated by centrifugation on a Ficoll-Hypaque gradient (Lymphoprep, Nygaard and Co., AG, Oslo, Norway). Cells were washed twice in phosphate-buffered saline (PBS) (GIBCO, Grand Island, NY) and once in medium RPMI 1640 (GIBCO).

Separation of T and B Cells

T cells were isolated by rosetting with neuraminidase-treated sheep red blood cells as previously described.19-20 B cells were isolated either as EAC rosettes (sheep erythrocytes coated with antibody and complement)21 or by use of anti-immunoglobulin columns according to the method of Chess.22

Lymphocyte Culture

Lymphocytes were cultured as previously described22 in microtiter plates at a final concentration of 10^6 cells/ml in a volume of 0.2 ml RPMI 1640 medium, containing 20% heat-inactivated horse serum (GIBCO), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1% 200 mM glutamine in Falcon 3040 microtiter plates in an atmosphere of 5% CO₂ at 37°C for the time indicated. Phytohemagglutinin (PHA, purified, Burroughs Wellcome, Research Triangle Park, NC) was added at the optimal stimulatory concentrations of 2-5 μg/ml. Response was measured by [methyl-3H]-thymidine or [4-5 H]-leucine incorporation for the last 16 hr of culture. [3H]-thymidine (Schwarz-Mann, Orangeburg, NY, specific activity 1.9 Ci/mmol) was added to a final concentration of 0.2 μCi/well. Cells, 16 hr after addition of either compound, were collected with a

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RESULTS

As has been previously reported, mononuclear cells from patients with CLL showed a delayed and moderately decreased peak response to PHA (Fig. 1). The incorporation of [methyl-3H]-thymidine by normal cells peaked between 65 and 113 hr, with a maximum mean incorporation at 89 hr, while the highest counts were observed at 113 hr with CLL mononuclear cells (Fig. 1A). However, even at 113 hr, CLL mononuclear cells incorporated less thymidine than did normal cells at 65 and 89 hr. These differences in response pattern were also noted for the incorporation of [4-5, 3H]-leucine into protein (Fig. 1B).

Adenosine inhibited [methyl-3H]-thymidine incorporation to a greater extent in CLL than in normal
mononuclear cells; significant greater inhibition of the CLL lymphocytes was observed with initial adenosine concentrations between 50 and 1,000 µM (Fig. 2). This effect was apparent at 65, 89, and 113 hr of incubation. The stimulation of [methyl-3H]-thymidine incorporation noted in normal mononuclears when adenosine was added at initial concentrations of 50 and 100 µM[10] was not seen with CLL cells. When the effect of adenosine on leucine incorporation was determined (Fig. 3), significantly greater inhibition of PHA-induced stimulation of CLL cells as compared to normal mononuclear cells was again observed. The addition of uridine, which has previously been shown to “rescue” normal lymphoid cells[10,12] from adenosine toxicity, was equally effective in rescuing CLL and normal mononuclear cells (Fig. 4). The addition of uridine abolished the differential inhibition by adenosine of CLL as compared to normal cells.

The effect of compounds structurally related to adenosine was examined next (Fig. 5 and Table 1). As we have reported previously,[23] in the absence of adenosine deaminase inhibitors, 2'deoxyadenosine was less inhibitory than adenosine to leucine incorporation by normal mononuclear cells (Fig. 5). This also held true for CLL lymphocytes. The CLL cells were more inhibited by 2'deoxyadenosine than were normal cells. The greater inhibition of CLL cells was only demonstrable at the highest concentration of deoxyadenosine (Fig. 5). Three additional analogues were tested, although less extensively (Table 1). The analogue, 5'deoxyadenosine, which has been reported to be neither phosphorylated by adenosine kinase nor deaminated by ADA,[27] was as potent as adenosine in inhibiting leucine incorporation under these conditions. Similar effects were seen with 2'diaminopurine arabinoside. Of the compounds tested, 2'diaminopurine ribonucleoside appeared to be the most effective inhibitor
of leucine incorporation. Slightly greater inhibition of PHA-induced leucine incorporation by CLL cells was observed with all of the compounds.

To determine the relationship between ADA activity and adenosine-mediated inhibition, cells were cultured in the presence of varying concentrations of the ADA inhibitor, EHNA, with several concentrations of adenosine. EHNA alone (Table 2), in a concentration of up to 0.8 μM, was only moderately inhibitory, but enhanced the inhibitory effect of adenosine. This is seen most clearly at 100 μM adenosine with normal cells. The major effect of EHNA was a shift in the dose–response curve, showing an increased sensitivity to adenosine. In the presence of EHNA, the increased inhibition of CLL compared to normal cells was not apparent at 100 μM adenosine, but became noticeable at 10 μM adenosine. At the higher concentration of adenosine (100 μM), the presence of EHNA abolished the difference between CLL and normal cells.

The Ficoll-Hypaque mononuclear cell population from normal subjects contains a higher percentage of monocytes and T cells than their CLL counterparts. For this reason, the effect of adenosine on 3H-leucine incorporation of monocyte-depleted preparations was determined next. The results of these experiments revealed that the removal of mononuclear cells by attachment either to GWF or to carbonyl iron had no consistent significant effect on the extent of inhibition by adenosine (data not shown).

The effect of enrichment with B or T subpopulations on adenosine-mediated inhibition is shown in Table 3. In the typical experiment shown, a comparison of T-cell-enriched preparations from a normal subject with T-cell-enriched preparations from a patient with CLL shows an equal degree of inhibition by adenosine. In contrast, the B-cell-enriched CLL preparation was markedly more inhibited by adenosine than its normal counterpart.

The rate of disappearance of exogenous adenosine added to cultures of intact CLL and normal lymphocytes was investigated next. The results shown in Fig. 6 indicate that the disappearance of adenosine added to CLL cultures is markedly prolonged over that of normal control cultures. These results are consistent, at the cellular level, with earlier data obtained with cell-free extracts documenting lower ADA activity in CLL lymphocytes.

**DISCUSSION**

The results of the experiments reported above show that adenosine and 2’deoxyadenosine are more toxic in

### Table 1. Effect of Adenosine and Adenosine Analogues on the PHA Response

<table>
<thead>
<tr>
<th>Adenosine Concentration (μM)</th>
<th>CLL</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ar (n = 3)</td>
<td>19 ± 6</td>
<td>40 ± 17</td>
</tr>
<tr>
<td>5’dAr (n = 3)</td>
<td>0 ± 0</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>DAP (n = 2)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Ara DAP (n = 2)</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>100 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ar (n = 3)</td>
<td>49 ± 16</td>
<td>83 ± 14</td>
</tr>
<tr>
<td>5’dAr (n = 3)</td>
<td>59 ± 10</td>
<td>77 ± 9</td>
</tr>
<tr>
<td>DAP (n = 2)</td>
<td>8</td>
<td>52</td>
</tr>
<tr>
<td>Ara DAP (n = 2)</td>
<td>48</td>
<td>73</td>
</tr>
<tr>
<td>50 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ar (n = 3)</td>
<td>61 ± 9</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>5’dAr (n = 3)</td>
<td>71 ± 12</td>
<td>88 ± 10</td>
</tr>
<tr>
<td>DAP (n = 2)</td>
<td>22</td>
<td>74</td>
</tr>
<tr>
<td>Ara DAP (n = 2)</td>
<td>62</td>
<td>81</td>
</tr>
</tbody>
</table>

Values are expressed as percent ± SD of the PHA-induced, cycloheximide-sensitive, TCA-precipitable incorporation of [4-5]H-leucine. Ar, adenosine; 5’dAr, 5’deoxyadenosine; DAP, 2’6’-diaminopurine ribonucleoside; Ara DAP, 9β-o-arabinofuranosyl 2’6’-diaminopurine. All compounds were added at time 0 and cells harvested at 65 hr.

### Table 2. Effect of EHNA on Adenosine-Induced Inhibition of the PHA Response‡

<table>
<thead>
<tr>
<th>Adenosine Concentration (μM)</th>
<th>CLL</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHNA (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.8</td>
<td>84 ± 9</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>1.6</td>
<td>84 ± 8</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>8.0</td>
<td>69 ± 7</td>
<td>73 ± 4</td>
</tr>
</tbody>
</table>

* ‡p < 0.01, comparing CLL mononuclears to NL.
‡ Values are expressed as percent of the PHA-induced, cycloheximide-sensitive, TCA-precipitable increased incorporation of 3H-leucine, ± SD.
Number of experiments = 3.
ADENOSINE EFFECTS ON CLL LYMPHOCYTES

Fig. 6. Degradation of \(^{14}\)C-adenosine by intact, PHA-stimulated lymphocytes from normal subjects and patients with CLL. See Materials and Methods for experimental conditions.

vitro to CLL than to normal lymphocytes. Although the data are not as extensive, three additional adenosine analogues also appear to be more toxic for CLL cells in vitro. This differential toxicity was observed in mononuclear cell preparations consisting of lymphocytes and monocytes, as well as with preparations depleted of monocytes. The question arose as to whether the increased sensitivity of CLL lymphocytes reflected a characteristic of the CLL B lymphocytes or stemmed from the relative paucity of T cells in the CLL preparations as compared to that of normal lymphocyte subpopulations. Results of experiments with the enrichment of CLL or depletion of normal lymphocyte preparations of T cells suggest that the difference in response is not explained by the altered B/T ratio in CLL. What appears to be the case is that CLL B cells are more susceptible to the adenosine toxicity than normal B-cell-enriched populations.

The CLL B cell may be unusually susceptible to the toxic effect of adenosine in vitro because of the low level of ADA in these cells. Thus, because of the differences in the rate of deamination of adenosine, CLL lymphocytes are actually exposed to higher levels of adenosine for longer time periods than are the normal cells. This interpretation is not supported by the finding that high concentrations of ADA inhibitors do not abolish the differential susceptibility of CLL cells to adenosine toxicity, but simply lower the concentration of adenosine at which this differential toxicity is seen. Although the increased length of exposure to adenosine may primarily explain the differential susceptibility of CLL to adenosine, this may not be the sole mechanism.

Evidence supporting an additional mechanism for the susceptibility of CLL cells to adenosine nucleoside toxicity is provided by the inhibitory effect of 5’deoxyadenosine. This compound, which reportedly can neither be deaminated nor phosphorylated, but has recently been shown to be a substrate for 5’methylthioadenosine phosphorylase, was also a relatively potent differential inhibitor. This finding might be consistent with a more direct interaction with an “adenosine binding protein” such as s-adenosyl homocysteine (SAH) hydrolase or “adenosine receptors” affecting cyclic AMP concentrations. Alternatively, 5’deoxyadenosine toxicity may involve other pathways, as has been reported for Ehrlich ascites tumor cells.

Another mechanism that was considered relates to the low level of 5’nucleotidase (5’N) in CLL lymphocytes. While in lymphoid cell lines, an inverse relationship has been reported between 5’N levels and susceptibility to deoxyadenosine toxicity via dATP accumulation, this seems an unlikely explanation in CLL, since no difference was observed between the accumulation of dATP in CLL lymphocytes with low or high 5’N incubated with deoxyadenosine. Increased uptake of adenosine by CLL lymphocytes was also considered, but earlier work from this laboratory has not revealed differences in rates of uptake between normal and CLL lymphocytes. Regardless of the mechanism underlying the differential susceptibility of normal and CLL lymphocytes, these studies would suggest that the clinical evaluation of deoxycoformycin may be warranted in this disorder. Such studies have already been reported in acute leukemia.

Finally, it was noted that not only did the addition of uridine result in the partial rescue of both cells from adenosine toxicity, but it was also capable of abolishing the difference between normal and CLL cells in the residual remaining inhibition. Since abundant evidence supports the depletion of pyrimidine pools for one of the mechanisms of adenosine toxicity in vitro, a comparison of the effect of adenosine on the endogenous pyrimidine pools and phosphoribosyl pyrophosphate content of CLL and normal lymphocytes would be of interest.

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