Sensitivity of Leukemic Human Null Lymphocytes to Deoxynucleosides

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ABSTRACT

The growth of cultured leukemic T-lymphocytes is readily inhibited by deoxynucleosides, particularly thymidine, deoxyguanosine, and deoxyadenosine. By contrast, Epstein-Barr virus-transformed B-lymphocytes are relatively resistant to deoxynucleosides. Growth inhibition correlates with the development of high deoxyribonucleoside triphosphate pools following exposure to deoxynucleosides. Leukemic T-lymphocytes are deficient in ecto-5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity, and it has been postulated that deficiency of this enzyme decreases the capacity of these cells to degrade deoxyribonucleotides, rendering them sensitive to deoxynucleosides.

We find that the sensitivity of cultured null-type leukemic lymphocytes to growth inhibition by deoxynucleosides is similar to that of T-cells. However, the null cells contain normal levels of ecto-5'-nucleotidase. We infer that ecto-5'-nucleotidase deficiency does not have a central role in determining the deoxynucleoside sensitivity of leukemic lymphocytes.

INTRODUCTION

The recently discovered deficiencies of adenosine deaminase and purine nucleoside phosphorylase associated with inherited immunodeficiency disease have stimulated investigation of purine nucleotide metabolism in human lymphocytes (2, 3). These syndromes reflect absence of catabolic routes for purine deoxynucleosides associated with deoxyribonucleotide accumulation which subsequently inhibits DNA synthesis. These clinical syndromes indicate a specific vulnerability of lymphocytes, particularly T-cells at some stage in their differentiation, to dNTP2 intoxication (2, 3). More recently, it has been discovered that cultured T-type leukemic lymphocytes are extremely sensitive to growth inhibition by deoxynucleosides, particularly thymidine, deoxyguanosine, and deoxyadenosine (1, 4, 11). These cell lines have been shown to accumulate and sustain high levels of dNTP which appear to act as feedback inhibitors of ribonucleotide reductase, resulting in inhibition of DNA replication. It has been shown that long-term cultured T-lymphocytes as well as T-lymphocytes isolated directly from leukemic patients have deficient ecto-5'-nucleotidase activity (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) (1, 6, 11). It has been postulated that these cells have decreased capacity to degrade dNTP, making them particularly susceptible to deoxynucleoside toxicity because they rapidly accumulate toxic levels of dNTP (1, 11). We now show that the sensitivity of cultured leukemic null cell lines to deoxynucleosides is similar to that of T-cells. However, the null cell lines have ecto-5'-nucleotidase activities comparable to those of deoxynucleoside-insensitive B-cell lines. We infer that ecto-5'-nucleotidase does not have a central role in determining deoxynucleoside sensitivity of leukemic lymphocytes.

MATERIALS AND METHODS

Cell Lines and Culture. Cultured human leukemic lymphocytes derived from patients with various types of acute lymphoblastic leukemia and lymphocytes transformed by EBV were kindly provided by Dr. H. Lazarus (Sidney Farber Institute, Boston, Mass.), J. Minowada (Roswell Park Memorial Institute, Buffalo, N. Y.), I. Jack (Royal Children's Hospital, Melbourne, Victoria, Australia), and H. Zola (Flinders Medical Centre, Adelaide, South Australia).

The normal peripheral blood lymphocyte lines transformed by EBV were JE-Tg, WIL, GK, and LAZ007. The lines derived from patients with acute lymphoblastic leukemia were: (a) T-cell leukemia, CCRF-CEM, 8402, CCRF-HSB, and HPB-MLT; (b) null cell leukemia, REH and KM-3. The origin and characteristics of these malignant cells have been previously described and summarized by Minowada (6).

All cell lines were grown in suspension culture using Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum. The doubling times of these cell lines (24 to 30 hr) were similar.

Isotopes and Chemicals. [3H]dTTP, [3H]ATP, and [14C]AMP were purchased from the Radiochemical Centre, Amersham, England. Unlabeled dTTP, dCTP, dGTP, dATP, dTMP, AMP, thymidine, deoxyguanosine, deoxycytidine, and deoxyadenosine were obtained from Sigma Chemical Co., St. Louis, Mo., and P-L Biochemicals, Milwaukee, Wis. Polyethyleneimine cellulose thin-layer chromatography plates were obtained from E. Merck AG, Darmstadt, Germany. Micrococcus luteus DNA polymerase and template for the DNA polymerase assay for deoxythymidylate, the alternating copolymer of deoxycytidine and deoxythymidylate, and the ADP analog α,β-methyleneadenosine diphosphate was obtained from Miles Laboratories, Elkhart, Ind.

Deoxynucleoside Inhibition of Cell Growth. Cells in log growth diluted with fresh medium to 2 × 106/ml and 2-naliquots dispensed into Linbro multiwell tissue culture plate (Linbro Scientific, Inc., Hamden, Conn.). Deoxynucleosides (5 mM to 0.1 μM) were added, and after 120 hr cells were counted using a Linson Celloscope 401 electronic cell counter. Th percentage of inhibition of growth was then calculated, and the 50% inhibitory dose was determined (4).

Ecto-5'-nucleotidase Assay. This was measured by a modification of the method described by Wortmann et al. (11). Incubations were performed in duplicate at 37°C in a reaction volume of 50 μl containing 0.1 to 0.2 × 106 intact lymphocytes. The reaction mixture consisted of Roswell Park Memorial Institute Medium 1640 containing 0.5 mM [5-14C]AMP (0.05 μCi) or [β-3H]dTMP (0.05 μCi). The mixture was incubated for 15 min (B- and null cells) or 60 min (T-cells). The reaction was termi-
nated by heating at 80° for 5 min. The cells were then pelleted by centrifugation. Five μl of supernatant were spotted with cold adenosine and AMP or thymidine and TMP onto polyethylene-imine cellulose thin-layer chromatography plates. Nucleosides and nucleotides were separated by elution with methanol/water (1:1). The 2 UV-absorbing spots were cut out, and radioactivity was counted in a liquid scintillation counter.

**Breakdown of dTTP by Whole Cells.** Cells (2 x 10^6 ml) were incubated with 60 μM thymidine for 3 hr, spun down, and resuspended in fresh warm media. The dTTP pool was measured in the cells (a) prior to addition of thymidine, (b) at the end of the 3-hr incubation with thymidine, (c) at 20, 40, and 60 min after resuspending in fresh medium. The dTTP concentration in the cells was determined using the DNA polymerase assay (9). This was performed as previously described by us except that cells were extracted with 60% ethanol rather than 60% methanol (10). Following incubation with thymidine, 5 x 10^6 cells were collected by centrifugation at 4° at 300 x g and washed once in cold phosphate-buffered saline, pH 7.4 (Flow Laboratories, Rockville, Md.). To the cell pellet was added 1 ml of 60% ethanol, and the suspension was stored at -20° overnight to extract the nucleotides. The supernatant was collected and dried using a rotary evaporator. The extract was then diluted in a known volume of water and stored at -20° until assayed. The alternating copolymer of deoxyadenylate and deoxymethylidate (0.05 unit) was used as the template for assaying dTTP in a final assay volume of 200 μl. Incubation was carried out for 35 min, and 0.3 unit of DNA polymerase (M. luteus) was used. Standard curves were linear up to 50 pmol. The results were expressed as pmol/10^6 cells and are the means of duplicate assays.

**RESULTS**

**Deoxynucleoside Sensitivity of Cultured Human Lymphocytes.** The growth of malignant T- or null-cell lymphocytes was found to be highly sensitive to thymidine and deoxyguanosine, less sensitive to deoxycytidine, and comparatively insensitive to deoxycytidine. By contrast, cultured peripheral blood B-lymphocytes transformed with EBV were relatively resistant to inhibition by all deoxynucleosides with the exception of deoxyguanosine (50% inhibitory dose, 0.8 mM). Even for deoxyguanosine, they were more resistant than were the T- or null-cell lines (Table 1).

**Ecto-5'-nucleotidase Activity.** The levels of ecto-5'-nucleotidase activity in the leukemic T-cell lines range from undetectable to 4 nmol of substrate converted per 10^6 cells per hr when AMP was used as a substrate. When dTMP was used as a substrate, activity levels were below the sensitivity limit of the assay (Table 2).

The null leukemic lines had levels of 5'-nucleotidase activity which were considerably higher than those of the T-cell lines and similar to those found for the deoxynucleoside-resistant EBV-transformed B-cell lines. No significant difference in activity was found when AMP or dTMP were used as substrates (Table 2).

To determine whether the AMP- and dTMP-degrading activity detected in the intact lymphocyte assay using both null- and B-cell lines was specific for ecto-5'-nucleotidase or represented a broad-spectrum phosphatase, we used the ADP analog α,β-methylene-adenosine diphosphate. This analog inhibits ecto-5'-nucleotidase but not nonspecific phosphatases. α,β-Methylene-adenosine diphosphate at a concentration of 3 mM inhibited degradation of AMP and TMP by both the null-cell and B-cell lines (Table 3).

**Breakdown of dTTP by Whole Cells.** Following incubation with thymidine and subsequent washing and resuspension in fresh medium the dTTP levels rapidly returned to preincubation levels in the deoxynucleoside-insensitive EBV-transformed B-cell line (JE-Tg). This contrasted with the delayed rate of fall in the sensitive T-cell line (CCRF-CEM) and the sensitive null cell lines (KM-3 and REH) (Chart 1). Similarly, following incubation with thymidine, the relative increase in the dTTP pool was greater in T-cell and null-cell lines than in B-lymphocyte lines.

**DISCUSSION**

The mechanism of deoxynucleoside-induced growth inhibition appears to reflect the ability of a cell to achieve and maintain a high concentration of the triphosphate of the inhibit-
that a low 5'-nucleotidase activity allowed cells to accumulate toxic levels of deoxyribotriphosphates. The role inferred for 5'-nucleotidase was a rate-limiting step in the degradation of deoxyribonucleoside monophosphates, thereby also regulating the level of dNTP within the cell (1, 11). We found that dTTP levels in deoxynucleoside-sensitive T- and null cells slowly decay at similar rates, compared to the rapid fall in a resistant B-cell line (Chart 1).

The finding that deoxyribonucleoside-sensitive null leukemic cells have levels of 5'-nucleotidase similar to those of deoxynucleoside-resistant B-cell lines indicate that at least in these cells decreased decay rates of dTTP are not due to deficiency in ecto-5'-nucleotidase. Reaman et al. (7) have studied ecto-5'-nucleotidase activity of leukemic cells collected from the peripheral blood of 23 patients with newly diagnosed acute lymphoblastic leukemia. They found a substantial decrease in ecto-5'-nucleotidase activity in cells with T markers. This contrasted with the higher activity found in null-type leukemic cells. The activity in these cells was comparable to those of normal lymphocytes. Our finding of normal levels of ecto-5'-nucleotidase in cultured leukemic null cells indicates that this surface marker is not lost during the transition to a permanent cultured cell line.

The exact physiological role for ecto-5'-nucleotidase has not been elucidated. A simplistic function is the conversion of nucleotides to nucleosides which are then transported across the cell plasma membrane. The finding of normal levels of ecto-5'-nucleotidase activity in cultured null leukemic lymphocytes which are deoxynucleoside sensitive suggests that ecto-5'-nucleotidase deficiency does not have a central role in determining deoxynucleoside sensitivity.

It should be noted that the toxicity of deoxyadenosine to cultured cells and the manifestations of adenosine deaminase deficiency may be nucleotide independent. It has been shown that deoxyadenosine inactivates S-adenosylhomocysteine hydrolase and that S-adenosylhomocysteine accumulates (5). However, the analogy between toxicity of deoxynucleosides to cultured T- and null cells and the predominant T-cell defects in adenosine deaminase and purine nucleoside phosphorylase deficiency is striking. It does appear worthwhile to attempt to define a biochemical phenomenon unique to T-cells (and null cells) unifying the dNTP accumulation in malignant T- and null cells and the inborn errors of purine catabolism associated with immunodeficiency.

REFERENCES

Deoxynucleoside Sensitivity of Leukemic Null Cells

Table 3

<table>
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<th>Cell lines</th>
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Chart 1. Breakdown of dTTP in intact T-, B-, and null cells. Cells in log-phase growth (2 × 10⁴/ml) were incubated with 50 μM thymidine for 3 hr, centrifuged, and resuspended in fresh warm thymidine-free media. The dTTP pool was measured in the cells at 20, 40, and 60 min after resuspension in the fresh medium. A, T-cell line (CCRF-CEM); B, EBV-transformed B-cell (JE-Tg); C, null-cell line (REH); D, null-cell line (KM-3).

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