ABSTRACT

Cultured leukemic T-lymphoblasts, incubated in the presence of inhibitors of adenosine deaminase, are exquisitely sensitive to growth inhibition by deoxyadenosine. An analogy between this phenomenon and human combined immunodeficiency disease associated with inborn adenosine deaminase deficiency and the use of inhibitors of adenosine deaminase in the management of T-cell acute lymphoblastic leukemia has been noted. These phenomena are believed to reflect accumulation of high intracellular concentrations of deoxyadenosine triphosphate (dATP) following phosphorylation of deoxyadenosine, inhibiting replicating T-cells. In an attempt to extend these observations to noncultured, nonleukemic T-cells, we studied deoxyadenosine metabolism in human thymocytes. Human thymuses were separated into large replicating and small nondividing cell types by centrifugal elutriation. Both thymocyte subpopulations elevated their dATP pools on incubation with μM concentrations of deoxyadenosine in the presence of erythro-9-[3-(2-hydroxynonyl)]adenosine, an inhibitor of adenosine deaminase. These dATP pool rises were similar in extent to those found in cultured leukemic T-lymphoblasts. However, the finding that small nonreplicating thymocytes elevate their dATP pool was unexpected. This prompted study of unstimulated peripheral blood lymphocytes. These cells (T and non-T) showed a similar elevation of their dATP pool on incubation with deoxyadenosine. Furthermore, these nondividing peripheral blood lymphocytes were killed by μM concentrations of deoxyadenosine in the presence of an inhibitor of adenosine deaminase. The biochemical mechanism of this G_{2}-phase cell death is not known.

These findings provide impetus for the investigation of adenosine deaminase inhibitors as lympholytic immunosuppressants or as agents cytotoxic to noncycling malignant lymphoid cells.

INTRODUCTION

Recently, drugs capable of inhibiting ADA\(^2\) (EC 3.5.4.4) have been introduced into clinical trial in the management of acute lymphoblastic T-cell leukemia. Patients with T-cell leukemia treated with deoxycoformycin, an inhibitor of ADA, have undergone dramatic remissions (23, 33, 47) associated with the development of lymphopenia. However, patients with nonleukemic lymphological cancer treated with deoxycoformycin also developed lymphopenia (36).

Similarly, the inherited deficiencies of the purine catabolic enzymes ADA and PNP (EC 2.4.2.1) are associated with severe immunodeficiency disease in children. Individuals with ADA deficiency have impaired T- and B-lymphocyte function (18), while those with PNP deficiency have primarily a defect in T-cell function (5, 17, 39). Both conditions are associated with thymic involution and profound lymphopenia. The presence of high levels of dATP in ADA-deficient erythrocytes (7, 9) and of dGTP in PNP-deficient erythrocytes (6) has implicated these metabolic products as effectors of purine nucleoside toxicity.

The significance of dATP toxicity was further emphasized when restoration of immune function in an ADA-deficient child was seen to accompany a reduction in erythrocyte dATP levels after transfusion with normal ADA-containing erythrocytes (7).

It was proposed that lymphotoxicity might be the result of allosteric inhibition of ribonucleotide reductase by these triphosphates with a consequent depletion of other dNTP and inhibition of DNA synthesis (3, 45). Such hypotheses are based on studies demonstrating elevations in dATP pools correlating with sensitivity to μM concentrations of dAdo by cultured human T- and null-cell leukemic lymphoblasts in vitro, while cultured EBV-transformed B-lymphoblasts show sensitivity and dATP pool elevations only at mM concentrations (4, 14, 46).

However, the lymphopenia of patients with ADA deficiency and the prompt development of lymphopenia in patients treated with ADA inhibitors (deoxycoformycin) suggest the possibility that nondividing lymphocytes are sensitive to dAdo. We report an investigation of the influence of dAdo in the presence of an inhibitor of ADA on human thymocytes and unstimulated human PBL. We show that both replicating and nonreplicating thymocytes freshly isolated from human thymuses elevate their dATP pools following exposure to μM concentrations of dAdo. We also demonstrate that human PBL similarly elevate their dATP pool. In addition, μM concentrations of dAdo in the presence of an inhibitor of ADA are capable of killing these nondividing cells. This implies a biochemical mechanism of dAdo toxicity which is independent of inhibition of ribonucleotide reductase and inhibition of DNA replication.

MATERIALS AND METHODS

Reagents

Tritiated dTTP, dCTP, dGTP, and dATP were obtained from the Radiochemical Centre, Amersham, England. Unlabeled dNTP, deoxyribonucleosides, and bovine pancreatic DNase I were obtained from Sigma Chemical Co., St. Louis, Mo. PEI plates were obtained from Merck, Darmstadt, Germany. Micrococcus luteus DNA polymerase, alternating copolymer of deoxyadenylate and deoxycytidylate, and alternating copolymer of deoxynosinylate and deoxycytidylate were obtained from Miles Laboratories, Inc., Elkhart, Ind. EHNA was a kind gift of Dr. C. Nichol, Burroughs Wellcome Co., Research Triangle

\(^{1}\) To whom requests for reprints should be addressed.

\(^{2}\) The abbreviations used are: ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; dNTP, deoxyribonucleoside triphosphates; dAdo, deoxyadenosine; EBV, Epstein-Barr virus; PBL, peripheral blood lymphocytes; EHNA, erythro-9-[3-(2-hydroxynonyl)]adenosine; RPMI-1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; E-rosetting, cells binding sheep erythrocytes; dNTP, deoxyribonucleoside triphosphates; dNTP, deoxyribonucleoside diphosphates; dNMP, deoxyribonucleoside monophosphates; dThd, thymidine; dGuo, deoxyguanosine; dCyd, deoxycytidine.

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Laboratories, North Ryde, N. S. W.

Human Lymphocytes. PBL were obtained from healthy blood donors. Mononuclear cell suspensions were obtained after Ficoll-Hypaque gradient centrifugation, and monocytes were depleted in 150-x 150 cm polystyrene flasks (37°, 2 h). E-rosetting T-cells and non-E-rosetting non-T-cells were obtained by standard methods (26) and separated by centrifugation on Ficoll-Hypaque. E-rosetting populations contained >90 to 96% E-rosetting cells, whereas the non-E-rosetting population contained <8 to 10% E-rosetting cells. Sheep erythrocytes were removed by hypotonic lysis in 85 mM Tris-HCl, pH 7.2, containing 80 mM ammonium chloride.

Human Thymocyte Subpopulations. Human thymus tissue was obtained from children aged 2 to 24 months with no known immune dysfunction who were undergoing elective cardiac surgery requiring partial thymectomy. A cell suspension was prepared by gently syringing finely minced tissue through 200-gauge wire gauze in RPMI-1640. The cell suspension was washed once in RPMI-1640 with 20 µg DNase I per ml. Thymocytes (2 to 3 x 10⁶) were loaded into a Beckman JVC-6 centrifuge fitted with a Beckman elutriator rotor with a conical chamber at 1500 rpm at ambient temperature with the centrifuge lid open and eluted in RPMI-1640 with fetal calf serum and 5 mM 2-naphthol-6,8-disulfonic acid (Tokyo Kasei Kogyo Co., Ltd., Japan) using a Cole-Parmer Masterflex pump with a Model 7013 pump head. Seven successive 100-ml fractions were collected at increments of 1 ml/min from a starting pump speed of 5 ml/min. Samples were washed free of eluting medium and resuspended in fresh RPMI-1640 immediately after collection.

Cultured Human Lymphocyte Lines. Cultured human leukemic lymphocytes derived from patients with acute T-lymphoblastic leukemia were provided by Dr. J. Minowada (Roswell Park Memorial Institute, Buffalo, N. Y.). These lines were CCRF-CEM, 8402, CCRF-HSB, and HPB-MLT. The origin and characteristics of these malignant cells have been described previously and summarized by Minowada (28).

Cultured diploid B-lymphocytes transformed by EBV were provided by Dr. H. Lazarus (Sidney-Farber Institute, Boston, Mass.), I. Jack (Royal Children’s Hospital, Melbourne, Vic.), and H. Zola (Flinders Medical Centre, Adelaide, S. A.). These lines were Je-Tg, WIL, JP, and LAZ-007.

All cell lines were grown in suspension culture in RPMI-1640 supplemented with 10% fetal calf serum. The lines had approximately similar doubling times (24 to 30 hr) and were studied in the log phase of growth.

Flow Cytometry

Light Scatter Analysis. Cells (5 x 10⁶/ml) were suspended in RPMI-1640 supplemented with 10% fetal calf serum and stained with fluorescein diacetate (2.5 µg/ml). Measurements of narrow angle laser light scatter (between 14° and 13°) were made using a Becton Dickinson (Mountain View, Calif.) FACS III fluorescence activated cell sorter. Narrow angle light scatter of viable cells closely correlates with cross-sectional cell area and, as mammalian cells in suspension usually adopt a spherical shape, therefore reflects cell volume. Cell samples were stained before analysis with fluorescein diacetate (50 µl of fluorescein diacetate [50 µl of fluorescein diacetate (50 µg/ml) to 10⁶ cells]. As fluorescein is only retained in viable cells with a intact membrane (34), the light-scattering properties of debris and dead cells, which have little fluorescence, can be electronically gated out. Values representing the histogram mean were then calculated from histograms of cellular light scattering. Upper and lower limits of subpopulations of large and small thymocytes were determined from these histograms by eye, and the respective population proportions were determined by computer (27).

DNA Analysis. Cellular DNA content was measured using a Model ICP22 flow cytometer (Ortho Instruments, Westwood, Mass.), and cells were stained with ethidium bromide and mithramycin as described previously (41, 42).

Incubation of Lymphocytes in dAdo

PBL (1 x 10⁶/ml) were incubated at 37° in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered RPMI-1640 supplemented with glutamine (0.2 mM), penicillin (100 µg/ml), streptomycin (100 units/ml), and 10% extensively heat-inactivated fetal calf serum (61°, 4 hr) in 25-ml polystyrene tissue culture flasks. After a 30-min preincubation in 5 µM EHNA, dAdo was added to duplicate cultures at the concentrations indicated, and the cultures were sampled at designated times. A minimum of 600 cells was hand counted in a hemocytometer, and the number of live and dead cells was determined by phase-contrast microscopy and trypan blue exclusion.

Intracellular dNTP Measurements

Cells (1 to 2 x 10⁶/ml) were incubated as above in various concentrations of deoxyribonucleosides. Cells were preincubated in 5 µM EHNA for 30 min before adding dAdo. Cells were washed once in Dulbecco’s phosphate-buffered saline [NaCl (0.8 g/liter-KCl (0.02 g/liter)]-Na₂HPO₄ (0.115 g/liter)-K₂HPO₄ (0.02 g/liter), pH 7.2] containing 2 mM EDTA and extracted overnight in 60% ethanol (−20°). dNTP pools were measured by a modification of the DNA polymerase assay (24, 38) as described previously (40).

Incorporation of labeled nucleotide into the alternating copolymer of deoxyadenylate and deoxythymidylylate and the alternating copolymer of deoxynosinylate and deoxycytidylylate chains reached a stable plateau after a 30-min incubation for extracts of all cell types so that extent of incorporation of label was proportional to the limiting dNTP. Assays were routinely terminated at 60 min. Recovery of added dNTP standards was 85 to 95% for extracts of all cell types. Extracts of each cell type were free of nuclease activity on testing under assay conditions by the method of North et al. (31). When dNTP standards were added to ethanol extracts of each cell type, activities were found to be one-third that of equimolar additions of dNTP standards, despite negligible reaction with dNTP standards in the absence of cell extracts. The addition of dNMP standards to cell extracts had no effect on the assay activity. Thus, dNMP kinase but not dNMP kinase activity was demonstrated in the 60% ethanol extracts (31). We have found a high correlation (90 to 105%) between dNTP values measured by the DNA polymerase method and those assayed by high performance liquid chromatography on the same cell extracts where these values are sufficiently high to permit the latter assay. In addition, the absence of detectable nuclease or dNMP kinase activities in these extracts makes it unlikely that degradation and reutilization of template, as suggested for methanol extracts of HeLa cells by North et al. (31), would result in large artificial elevations in measured deoxynucleotides by this assay.

dThd Kinase, dThd Phosphorylase, and Ecto-dThd Triphosphatase. These enzymes were assayed by methods described previously (13).

Protein. Protein was assayed by the method of Lowry et al. (25) with bovine serum albumin as standard.

RESULTS

Subfractionation of Human Thymocytes by Elutriation. Light scatter analysis of unfractionated thymocytes revealed a skewed distribution of cell volumes, indicating the presence of a population of large cells accounting for 10 to 18% of the total cell number. Cytodensitometric analysis of the DNA content of unfractionated thymocytes from 6 separate thymuses demonstrated an overall proliferative index (i.e., percentage of S + G₂ + M) of 18 to 23%. In thymuses from the fetus or very
young children, the proportion of larger cells and the proliferative index were higher. Following elutriation, the cell recovery was 85 to 90%. Viability assessed by phase-contrast microscopy and trypan blue exclusion was greater than 98% in all fractions collected.

Fraction 1 was found to contain contaminating erythrocytes and cell debris and was discarded. Fraction 2 contained 87 to 98% small thymocytes and was used as the "small thymocyte subpopulation" (Chart 1A). The greatest enrichment of large cells occurred in Fractions 6 and 7 (30 to 36%). These fractions were combined and reelutriated. The seventh fraction from this second elutriation, which contained 35 to 50% large cells, was used as the "large thymocyte subpopulation" (Chart 1B). DNA histograms gave a proliferative index of 3 to 10% in the small thymocyte fraction compared with 30 to 50% in the large cell fraction (Chart 1, C and D).

Effect of Deoxyribonucleosides on Thymocyte dNTP Pools. Base line dNTP pools for fractionated human thymocytes and cultured lymphoblasts are given in Table 1. Following incubation of both small nonproliferating and large proliferating thymocytes with dAdo (4 hr in the presence of 5 μM EHNA), there was an elevation in the dATP pool which was similar in both cell types (Chart 2). The extent of this dATP pool elevation was dependent on the concentration of dAdo. dAdo at 1 μM elevated the dATP pool some 2-fold, while at 10 μM dAdo, the pool elevation was 4-fold. A similar dAdo concentration-dependent rise in dATP pool was found for the cultured leukemic T-cell line CCRF-CEM. A cultured EBV-transformed B-cell, LAZ-007, failed to elevate its dATP pool as has been reported previously (13) (Chart 2).

Similar responses were seen following incubation of thymocyte subfractions with dGuo (Chart 3). However, the CCRF-CEM line showed a greater elevation of its dGTP pool in comparison to the thymocytes. Again, the EBV-transformed B-cell line LAZ-007 did not elevate its dGTP pool until concentration of dGuo exceeded 300 μM.

In contrast, the small nonproliferating thymocytes did not elevate their dTTP pool following exposure to dThd (Chart 4). The large proliferating thymocytes elevated their dTTP at 100 μM dThd, and this pattern of dTTP response was similar to that of the EBV-transformed B-cell line (LAZ-007). In view of the

<table>
<thead>
<tr>
<th>Cells</th>
<th>dTTP</th>
<th>dATP</th>
<th>dGTP</th>
<th>dCTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large thymocytes</td>
<td>13.8 ± 6.6</td>
<td>11.9 ± 5.7</td>
<td>5.8 ± 2.2</td>
<td>5.7 ± 3.2</td>
</tr>
<tr>
<td>Small thymocytes</td>
<td>8.6 ± 5.4</td>
<td>4.4 ± 2.3</td>
<td>2.2 ± 1.0</td>
<td>3.9 ± 2.9</td>
</tr>
<tr>
<td>T-lymphoblasts</td>
<td>19.4 ± 5.4</td>
<td>26 ± 6.5</td>
<td>13.9 ± 4.6</td>
<td>11.2 ± 6.2</td>
</tr>
<tr>
<td>B-lymphoblasts</td>
<td>8.8 ± 4.7</td>
<td>3.9 ± 1.7</td>
<td>1.4 ± 0.4</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>PBL</td>
<td>1.4 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.05</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of 3 to 6 determinations.

Table 1

DNT pools in untreated lymphoid cells

Thymocyte suspensions were prepared from human infant thymuses and populations enriched for 87 to 95% small thymocytes, or 35 to 50% large thymocytes were prepared by centrifugal elutriation. PBL were prepared by centrifugation of whole blood from normal human donors on Ficoll-Hypaque and monocyte depletion in polystyrene tissue culture flasks. Cultured cells were grown in RPMI-1640 supplemented with 10% fetal calf serum and maintained in log growth. Cells were diluted with fresh medium to 2 x 10⁶/ml and grown for 24 hr. Cells were harvested by centrifugation and extracted with 80% ethanol, and the dNTP pools were measured by the DNA polymerase assay (38, 40).

**R. F. Kefford and R. M. Fox**
presence in the large thymocyte fraction of 50 to 70% small thymocytes, the extent of the dTTP pool elevation following dThd exposure of the large thymocytes may therefore be 2- to 3-fold underestimated. The rise in dTTP pool in the CCRF-CEM T-cell line has been reported previously (12, 13).

Cultured T-lymphoblasts which elevate their dTTP pools on incubation with dThd have a high dThd kinase, low dThd phosphorylase, and low level of ecto-dTTPase. This latter enzyme is capable of degrading dTTP (13). The EBV-transformed B-lymphoblasts have a slightly lower dThd kinase level, a higher dThd phosphorylase level (12), and high level of ecto-TTPase (13).

By contrast, the small thymocytes have an extremely low

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**Table 2**

<table>
<thead>
<tr>
<th>dThd-metabolizing enzymes</th>
<th>Thymidine kinase (nmol/mg protein/hr)</th>
<th>Thymidine phosphorylase (μmol/mg protein/hr)</th>
<th>Ecto-dTTPase (nmol/10^10 cells/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated thymocytes</td>
<td>0.87 ± 0.43^a</td>
<td>0.094 ± 0.051</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Small thymocytes</td>
<td>0.10 ± 0.04</td>
<td>0.081 ± 0.053</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Large thymocytes</td>
<td>1.37 ± 0.28</td>
<td>0.125 ± 0.049</td>
<td>0.21 ± 0.21</td>
</tr>
<tr>
<td>Cultured T-lymphoblasts^b</td>
<td>14.6 ± 0.8</td>
<td>0.034 ± 0.02</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cultured B-lymphoblasts^c</td>
<td>6.62 ± 0.5</td>
<td>0.24 ± 0.11</td>
<td>1.97 ± 1.32</td>
</tr>
</tbody>
</table>

^a Mean ± S.E. for 4 to 6 determinations.  
^b CCRF-CEM, 8402, CCRF-HSB, and HPB-MLT.  
^c Je-Tg, WII, JP, and LAZ-007.
level of dThd kinase, explaining their inability to elevate their dTTP pool despite low levels of dThd phosphorylase and ecto- dTTPase. It is not possible to clearly evaluate the dTTP pool response of large thymocytes to dThd due to the presence in this cell fraction of small thymocytes which have low levels of dThd kinase and inability to elevate their pools.

Influence of Deoxyribonucleosides on Unstimulated PBL Pools. The rise in purine dNTP following exposure of small nonproliferating thymocytes to dAdo and dGuo, respectively, was unexpected. This observation led us to carry out similar studies with unstimulated PBL. Base line dNTP pools in PBL are given in Table 1. Again, following incubation with dAdo, in the presence of 5 μM EHNA, there was elevation of the dATP pool (Chart 2). This was also dependent on the concentration of dAdo and was of a similar magnitude to that seen with the thymocytes and a cultured T-cell line. This ability of PBL to elevate their dATP pool following exposure to low concentrations of dAdo was not a unique T-cell phenomenon. Both T- and non-T-lymphocytes elevated dATP pools (Table 3). By contrast, the PBL showed less elevation of dGTP pools following exposure to dGuo (Chart 3). The dGTP pool only rose at 100 μM dGuo. There was no rise in the dTTP pool following exposure to dThd, even at 300 μM dThd (Chart 4).

dAdo Toxicity to Unstimulated PBL. The PBL were killed by incubation with dAdo in the presence of 5 μM EHNA. This toxicity was both time and dAdo concentration dependent (Chart 5). dAdo (1 μM) killed 50% of cells after 72 hr of incubation, and after 96 hr, only some 10% of cells remained viable, compared to 90% of control PBL treated with 5 μM EHNA alone. Higher concentrations of dAdo killed a greater percentage of cells but did not kill cells within the first 24 hr of incubation. We have not yet determined whether, at low dAdo concentrations, there is any preferential killing of either T-, B-, or null-cell lymphocytes. The toxicity of 1 μM dAdo was entirely prevented by coincubation with dCyd (50 μM) (Chart 5). When cells treated with 30 μM dAdo were coincubated with 50 μM dCyd, protection was only partial with 30% of cells alive at 96 hr, compared to 8% of cells treated with dAdo alone. Analysis of the DNA content of the cells by flow cytometry daily throughout the incubation period demonstrated that neither treated nor untreated cells entered into S phase (data not shown).

Effect of Long-Term Incubation in 1 μM dAdo on dATP Pools of PBL. The dATP pools of PBL incubated in 1 μM dAdo plus 5 μM EHNA showed a modest elevation in dATP pools (2-fold) at 24 hr, which persisted through the 72-hr incubation period (Table 4).

**DISCUSSION**

In an attempt to correlate observations made in patients with ADA and PNP deficiencies and investigations using cultured lymphocyte lines, we initially studied deoxynucleoside metabolism in human thymocytes. The large human thymocyte represents a dividing T-cell which can be studied independently of

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**Table 3**

<table>
<thead>
<tr>
<th>Exogenous nucleoside</th>
<th>Concentration</th>
<th>Lymphocytes</th>
<th>dNTP (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>T</td>
<td>1.5 ± 0.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Non-T</td>
<td>1.5 ± 0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>dAdo + 5 μM EHNA</td>
<td>T</td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-T</td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td>dGuo</td>
<td>T</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-T</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>dThd</td>
<td>T</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-T</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 determinations.
in vitro mitogenic transformation and leukemic- or culture-induced change.

Centrifugal elutriation is a rapid method of separating large numbers of thymocytes with high recovery rates and little loss in cell viability. By using a second elutriation to further purify the large cell fraction, a population enriched to more than 35% large cells can be obtained, while the small fraction is always greater than 86% pure. The enriched large cell population had a proliferative index (S + G2 + M) of 30 to 50% compared with only 3 to 10% for small cells. Thus, while the large cell population was not pure, the small cell population was highly enriched, and it would be difficult to attribute biochemical findings in these cells to the very small number of dividing cells present. The surface characteristics, patterns of mitogen response, and higher thymidine labeling index of the subpopulation of large thymocytes suggest that they represent maturing precursor T-cells (15, 16).

The changes in dATP and dGTP pools of dividing human thymocytes, when incubated with dAdo and dGuo, respectively, correlate with the dNTP responses of the cultured T-lymphoblast cell lines. These changes in dNTP pools in cultured T-lymphoblasts have correlated with growth inhibition by dAdo and dGuo (2) and with inhibition of DNA synthesis by dGuo in cultured thymocytes (8).

The small, nondividing thymocytes, which as a population are enriched for markers of mature T-cells (15, 16), showed an ability to achieve large elevations in their dNTP pools which was not anticipated. These cells, on the basis of flow cyttofluorimetric analysis, are clearly not replicating DNA. The rises in dATP and dGTP pools following incubation with dAdo and dGuo, respectively, indicate the presence of purine deoxyribonucleoside kinases. The inability of these cells to elevate their dNTP pools on exposure to dThd reflects the absence of dThd kinase. Furthermore, the resting levels of dNTP in small thymocytes were relatively high, particularly when compared to the levels in unstimulated PBL (30, 43), the majority of which are thymus derived.

The unexpected ability of small, nondividing thymocytes to elevate their purine dNTP pools prompted us to study PBL. In the presence of EHNA, these cells elevated their dATP after exposure to μM concentrations of dAdo to a similar extent as the thymocytes or cultured leukemic T-cell line. However, the PBL could only elevate their dGTP pool at dGuo concentration an order of magnitude higher than found with thymic or leukemic T-cells. The explanation for this discrepancy may reflect the high PNP levels in PBL compared to thymocytes or leukemia T-cells (3) or the cell cycle dependence of dGuo kinase (1).

That PBL can elevate their purine dNTP pools on exposure to dAdo and dGuo correlates with the presence of purine deoxyribonucleoside kinase in unstimulated PBL (3, 37). The inability of PBL to elevate dTTP pool was anticipated in view of the very low dThd kinase levels in these cells (32). Furthermore, the ability of PBL to elevate their purine dNTPs is not specific to the T-cell subset but occurs with non-T-cells. This contrasts with the inability of EBV-transformed B-lymphocyte lines to elevate their dNTP pools (2, 13, 46).

The toxicity of dAdo to unstimulated human PBL raises several questions about the mechanisms of dAdo toxicity based on studies using mitogen-stimulated lymphocytes and cultured leukemic or virally transformed cell lines, which have been reviewed recently (19). Hypotheses have included that (a) high dATP levels inhibit ribonucleotide reductase and thus DNA synthesis, and (b) dAdo "suicide inactivates" S-adenosylhomocysteine hydrolase inhibiting transmethylation reactions. A dAdo concentration as low as 1 μM kills PBL. This level approximates the serum concentration of dAdo measured in ADA-deficient patients and is less than that in the serum of patients treated with deoxycoformycin (21, 29).

Despite the ability of PBL to elevate their dATP pools following exposure to dAdo, it is clearly unlikely that inhibition of ribonucleotide reductase or an effect on DNA polymerase would account for cell death in these nondividing cells. Furthermore, at 1 μM dAdo, which kills PBL, the rise in dATP pool is modest, even after 72 hr. We have not excluded the possibility that dAdo is inactivating S-adenosylhomocysteine hydrolase in PBL (20). A lymphocytotoxic effect of dAdo independent of inhibition of DNA synthesis is supported by the early toxicity (<20 hr) induced by dAdo in mitogen-stimulated PBL (44). We have reported separately a G1-phase arrest in cultured human leukemic T-cells after exposure to dAdo (3 μM) in the presence of EHNA (11). Those cells in S, after exposure to dAdo, complete that S phase. This is not compatible with ribonucleotide reductase inhibition. This contrasted with the S-phase block observed in these cells following exposure to dThd or hydroxyurea, agents which inhibit ribonucleotide reductase. This G1-phase block was associated with a rise in the dATP pool without a fall in the dCTP pool. Both of these phenomena were prevented by coincubation with dCyd. It is possible that the Gi phase block induced in replicating T-lymphoblasts and the toxicity to unstimulated PBL reflect a common biochemical mechanism. dCyd protects unstimulated PBL from low-concentration dAdo toxicity. Since "protection" by replenishment of a lowered dCTP pool (following ribonucleotide reductase inhibition) is likely, the protection offered by dCyd may possibly reflect competition with dAdo for transport into the cell or for phosphorylation to dATP. A ribonucleotidase-independent effect of dAdo is also suggested by patterns of [3H]uridine incorporation into DNA and RNA in human fibroblasts treated with toxic levels of dAdo and hydroxyurea (10).

Thus, the biochemical process by which dAdo kills PBL is not known at this time. However, the toxicity is associated with an elevated dATP pool which could potentially interfere with ATP-dependent or other metabolic steps. Henderson et al. (19) have reviewed possible mechanisms of dAdo toxicity which include depletion of intracellular ATP or formation of a cyclic dAdo nucleotide (19). Indeed, a decreased erythrocyte ATP content in association with hemolysis has been reported following deoxycoformycin administration (35).

The in vitro sensitivity of nondividing PBL to low concentrations of dAdo provides a new model, independent of DNA replication or cell division, for investigating the mechanism of dAdo toxicity. This observation provides impetus for the clinical investigation of ADA inhibitors as lympholytic immunosuppressants or as agents cytotoxic to noncycling malignant lymphoid cells.

ACKNOWLEDGMENTS

We wish to acknowledge the kind cooperation of Dr. T. B. Cartmill and Dr. D. C. Johnson, Royal Alexandria Hospital for Children, Sydney, for provision of thymus glands, and the Red Cross Blood Bank, Sydney, for provision of blood. We also thank Edith Tripp for her assistance with biochemical assays, Dr. I.

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RESULTS

**β-Glucuronidase Activity of Colonic Contents.** Table 1 summarizes the activity of β-glucuronidase in the contents of the proximal and the distal halves of the colons of rats treated with AOM. All animals were given weekly s.c. injections of AOM 11 times and were autopsied 20 weeks after the last injection. W, week(s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Proximal half</th>
<th>Distal half</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>0 ± 1.7</td>
<td>12.83 ± 1.65</td>
</tr>
<tr>
<td>C-GAL diet</td>
<td>12.05 ± 9.68</td>
<td>12.12 ± 4.49</td>
</tr>
<tr>
<td>Group A</td>
<td>6.94 ± 4.03</td>
<td>8.14 ± 4.42</td>
</tr>
<tr>
<td>Group B</td>
<td>4.43 ± 3.07</td>
<td>4.55 ± 2.75</td>
</tr>
<tr>
<td>Group C</td>
<td>4.51 ± 1.48</td>
<td>5.57 ± 3.85</td>
</tr>
<tr>
<td>Group D</td>
<td>10.40 ± 9.68</td>
<td>5.29 ± 3.01</td>
</tr>
</tbody>
</table>

a Three weeks after the first injection of AOM.
b μg phenolphthalein per mg, wet weight, of colonic content per hr.
c Means ± S.D.
d After 11 weekly injections of AOM.
e At sacrifice (20 weeks after last injection).
f Significantly different from control diet group at each period (p < 0.05).
g p < 0.01.

**Tumor Induction.** Table 2 summarizes the incidence of tumors in the 4 groups of rats treated with AOM: Group A, 100%; Group B, 97%; Group C, 92%, and Group D, 100%. The average number of colon tumors per rat was 9.9 ± 6.3 in Group A (control group), 6.4 ± 4.5 in Group B, 3.1 ± 4.0 in Group C, and 11.1 ± 7.9 in Group D. Thus, the average number of colon tumors per rat was 9.9 ± 6.3 in Group A (control group), 6.4 ± 4.5 in Group B, 3.1 ± 4.0 in Group C, and 11.1 ± 7.9 in Group D. Thus, the average number of colon tumors was significantly suppressed in Group B (p < 0.05) and Group C (p < 0.001). The distribution of these neoplasms in the proximal and distal halves of the colon is shown in Table 2. Tumors occurred throughout the colon in Groups A and D, but the number of tumors was significantly reduced in the proximal half in Groups B (p < 0.001) and C (p < 0.001). Furthermore, in Group C, the average number of tumors in the distal half was significantly reduced (p < 0.02). About 60% of the tumors were polypoid, and polypoid or intramural tumors often caused intestinal obstruction, bloody stools, and intussusception. The diameter of the colon tumors varied from 0.1 to 3.1 cm, and the distribution pattern according to size was almost the same in each group. Carcinomatosis of the peritoneum was present in a few rats, but liver and lung metastases were not found. No tumors were found in other organs, and no tumors were found in the intestine or other organs of rats not given AOM.

**Histology.** The majority of the colonic tumors were well-differentiated tubular adenocarcinomas, and in a few cases, signet-ring cell carcinomas were found invading the lamina propria or the serosa. There were no histological abnormalities in the liver, kidney, or spleen of rats fed the C-GAL diet.

**DISCUSSION**

Several studies have indicated that dietary factors are most important in the etiology of colon cancer (9, 11, 12, 40, 47). The nature of the diet affects the composition of the intestinal bacterial flora, and bacteria can produce carcinogen or cocarcinogens. The nature of the diet affects the composition of the intestinal bacterial flora, and bacteria can produce carcinogen or cocarcinogens.
Purine Deoxynucleoside Toxicity in Nondividing Human Lymphoid Cells

Richard F. Kefford and Richard M. Fox


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