Purine and Pyrimidine Metabolism in Human T Lymphocytes

REGULATION OF DEOXYRIBONUCLEOTIDE METABOLISM*

(Received for publication, March 24, 1983)

Amos Cohen†, Jerzy Barankiewicz, Howard M. Lederman, and Erwin W. Gelfand
From the Division of Immunology, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

Purine and pyrimidine deoxyribonucleoside metabolism was studied in G1 and S phase human thymocytes and compared with that of the more mature T lymphocytes from peripheral blood. Both thymocyte populations have much higher intracellular deoxyribonucleoside triphosphate (dNTP) pools than peripheral blood T lymphocytes. The smallest dNTP pool in S phase thymocytes is dCTP (5.7 pmol/10⁶ cells) and the largest is dTTP (48 pmol/10⁶ cells), whereas in G1 thymocytes, dATP and dGTP comprise the smallest pools. While both G1 and S phase thymocytes have active deoxyribonucleoside salvage pathways, only S phase thymocytes have significant ribonucleotide reduction activity.

We have studied ribonucleotide reduction and deoxyribonucleoside salvage in S phase thymocytes in the presence of extracellular deoxyribonucleosides. Based on these studies, we propose a model for the interaction of deoxyribonucleoside salvage and ribonucleotide reduction in S phase thymocytes. According to this model, extracellular deoxycytidine at micromolar concentrations is efficiently salvaged by deoxycytidine kinase. However, due to feedback inhibition of deoxycytidine kinase by dCTP, the maximal level of dCTP which can be achieved is limited. The salvage of both deoxycytidine, whereas the salvage of other deoxyribonucleosides.

Disorders of deoxyribonucleoside metabolism are believed to result in the T lymphocyte toxicity associated with adenine deaminase and purine nucleoside phosphorylase deficiencies (1-7). Numerous studies have shown that T leukemic cells as well as immature T cells of the thymus are exquisitely sensitive to disturbances in purine and pyrimidine deoxyribonucleoside metabolism (3, 4, 8-10). Moreover, activities of several enzymes associated with deoxyribonucleoside metabolism change during T lymphocyte differentiation (3, 4, 10-17). Activities of deoxyribonucleotide degradation enzymes are low in T leukemic cells and in thymocytes (12-17), whereas the activity of the deoxyribonucleoside salvage enzyme, deoxycytidine kinase, is highest in thymocytes and decreases during T lymphocyte differentiation (15, 17). As a result, T leukemic cells and immature thymocytes have increased ability to salvage and maintain high intracellular deoxyribonucleoside levels in vivo (3, 4, 10, 14, 15). However, the role of this unique composition of purine and pyrimidine enzymes to the normal differentiation of T lymphocytes is not understood.

Reduction of all four ribonucleotides is catalyzed by a single enzyme ribonucleoside diphosphate reductase (18). Studies on the regulation of deoxyribonucleoside triphosphate levels led to the construction of a model for the regulation of ribonucleotide reduction (18, 19). Parts of this model have been confirmed in transformed cell lines (reviewed in Ref. 20); however, the regulation of deoxyribonucleotide synthesis may differ in vivo. Aberrant deoxyribonucleoside metabolism may be acquired during culture or transformation (21-23). Further, since deoxyribonucleosides are not included in culture media, in vitro studies of deoxyribonucleotides metabolism in cultured cell lines emphasize the contribution of ribonucleotide reduction and de-emphasize the contribution of deoxyribonucleoside salvage.

In order to elucidate possible role(s) for deoxyribonucleotides in the differentiation of T lymphocytes, we have compared purine and pyrimidine deoxyribonucleoside metabolism in immature G1 and S phase thymocyte populations to the metabolism of the more mature T lymphocytes from peripheral blood. We have studied the salvage of individual purine and pyrimidine deoxyribonucleosides. A model is suggested to explain the interaction of deoxyribonucleoside salvage with ribonucleotide reduction for the regulation of dNTP levels in intrathymic T lymphocytes.

EXPERIMENTAL PROCEDURES

Cell Preparations—Peripheral blood was obtained from healthy blood donors, and E-rosetting T lymphocytes were purified as described (15). Human thymocytes were obtained from children (age 6 months to 6 years old) with no known immune dysfunction who were undergoing cardiac surgery. Mononuclear cell suspensions were prepared as previously described (15).

Human Thymocyte Subpopulations—Thymocytes were separated...
into two subpopulations (small cells predominantly in G1, >95% and large cells with S and G2/M phases) by BSA-gradient centrifugation. A 35% (w/v) solution of BSA (Cohn Fraction V, Sigma Chemical Co., St. Louis, MO) was prepared in Tris-HCl buffer, pH 7.2. The pH was adjusted to 5.2, osmolality was adjusted to 360 mosm with NaCl, and the solution was sterilized by filtration (0.45-μm filter, Millipore Corp., Bedford, MA). Dilutions of the BSA stock solution were made with 0.15 M sodium chloride and 0.15 M phosphate buffer, pH 7.2. For separation, 1-2 × 10^6 thymocytes were suspended in 1-2 ml of RPMI 1640 (Ontario Cancer Institute, Toronto, Ontario), gently layered over 1 ml of 25% BSA in 12 × 75 mm plastic tubes (Falcon Plastics, Oxnard, CA), and centrifuged at 4°C for 30 min at 900 g (2000 rpm in a Sorvall RT 25C; Ivan Sorvall, Inc., Newton, CN). Cells were recovered in the pellet fraction and 10% in the interface fraction. The interface fraction contained large thymocytes (mean cell volume, 216 fl); 45% of cells were in the S phase of the cell cycle and 15% were in the G0 + M phase.

Cell Cycle Analysis—Thymocytes were fixed with 70% ethanol and RNA was degraded by RNase (30 μg/ml; Worthington Biochemical Corp., Freehold, NJ). The cell cycle was then stained with propidium iodide (50 μg/ml; Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. Cellular DNA content was measured by flow cytometry using the Epic V cell sorter (Coulter Electronics, Hialeah, FL). Data analysis to determine the percentage of cells in each cell cycle phase was performed with the aid of the EASY Cell software system using the PARA 1 program (Coulter Electronics).

Cell Volume Measurements—Measurements of cell volume were made using the Coulter Counter with attached Channelyzer (Coulter Electronics).

Incorporation of Purine and Pyrimidine Deoxyribonucleotides—Lymphocytes (1 × 10^6 cells) were incubated in 1 ml of RPMI 1640 medium containing 1% heat-inactivated fetal calf serum. The cells were preincubated for 1 h before the addition of the specified radioactive purine or pyrimidine deoxyribonucleoside and then incubated for 1-2 h at 37°C in a 5% CO2 humidified incubator. Linearity of incorporation under these conditions was maintained for at least 3 h and incorporation of radioactive deoxyribonucleotides did not exceed 10% of the total radioactivity added. At the end of the incubation period, deoxyribonucleotides were extracted by the addition of 0.4 M perchloric acid for 30 min on ice. After centrifugation, the supernatant was neutralized by extraction with a 0.5 M solution of Alamine 336 in Freon-TF (24). Purine and pyrimidine deoxyribonucleotides were separated by two-dimensional thin layer chromatography using polyethyleneimine-cellulose sheets as described by Crabtree and Henderson (25). The plates were prepared by attaching a wick and washing overnight with 18 M ammonium formate with 2% boric acid, pH 7.0, followed by 5% methanol. For drying, nonradioactive nucleotide markers and 20 μl of the radioactive extract were spotted and run overnight in 50% methanol to wash bases and nucleotides to the wick. Fresh wicks were attached and the plates were developed to 8 cm above the origin with 1.8 M ammonium formate with 2% boric acid, pH 7.0, followed by 3.3 M ammonium formate with 4.2% boric acid, pH 7.0. Development was stopped when the leading nucleotide marker was close to the wick. The wick was removed and the plates were immersed in methanol for 15 min. After drying a fresh wick was attached and the plates were developed in the second dimension up to 2.5 cm above the origin with 0.5 M ammonium formate, pH 3.4, then to 8 cm above the origin in 2.0 M sodium formate, pH 3.4, and finally up to the wick in 4.0 M sodium formate, pH 3.4. The spots were visualized with ultraviolet light and cut, and the radioactivity was counted. Incorporation of deoxyribonucleosides into phase of the cell cycle was calculated by summing the radioactivity incorporated into DNA and reutilization of the template takes place (29). Under the above conditions, the dNTP assay was linear between 0.5 and 20 pmol for dCTP and dGTP, to 75 pmol for dTTP, and to 100 pmol for dATP. The number of cells used in each dNTP assay was chosen to fit the linear range. The reproducibility of the dNTP assay was within 10% S.D. and was reproducible when fresh sample was compared to the same sample after 14-day storage at −20°C.

Reagents—[5-3H]Cytidine (21 Ci/mmol), [methyl-3H]thymidine (77 Ci/mmol), [5-3H]deoxycytidine (25 Ci/mmol), [8-3H]dATP (17 Ci/mmol), [methyl-3H]dATP (25 Ci/mmol), and [3H]dGTP (25 Ci/mmol) were purchased from ICN (Irvine, CA). Purine and pyrimidine nucleotides and nucleosides were purchased from Sigma Chemical Co. (St. Louis, MO). 2′-Deoxycoformycin was obtained from Parke Davis Co. (Ann Arbor, MI). Poly(dA,T), poly(dG,C), and Escherichia coli DNA polymerase I were purchased from Miles Chemical Co.

RESULTS

Studies were performed using three distinct cell populations: mature peripheral blood T lymphocytes, primarily in G1 (>97%), and two fractions of immature intrathymic T lymphocytes separated on BSA gradient, i.e., the pellet fraction containing small G1 thymocytes (95% in G0) and the interface fraction containing large S phase-enriched (45%) thymocytes.

Deoxynucleoside Triphosphate Levels in T Lymphocyte Populations—Intracellular pools of purine and pyrimidine dNTP in peripheral blood T lymphocytes, G1 thymocytes, and S phase-enriched thymocytes are summarized in Table I. Both thymocyte populations have higher deoxynucleoside triphosphate levels than mature peripheral blood T lymphocytes. The levels of dNTP in immature G1 thymocytes are especially striking when compared to the much lower levels found in the more mature peripheral blood T lymphocytes.
Lymphocyte populations were prepared, nucleotides were extracted, and deoxyribonucleoside levels were determined by the DNA polymerase method as described under "Experimental Procedures." Results represent the means of four determinations of peripheral blood samples and of 10 different thymi each done in duplicate (±S.D.).

### Table I

<table>
<thead>
<tr>
<th>Deoxyribonucleoside Triphosphates</th>
<th>Peripheral blood T lymphocytes</th>
<th>Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small (G_1 phase)</td>
<td>Large (S phase)</td>
</tr>
<tr>
<td>dATP</td>
<td>2.35 ± 1.7</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.39 ± 0.28</td>
<td>6.6 ± 3.0</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.30 ± 0.15</td>
<td>19.5 ± 6.3</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.24 ± 0.02</td>
<td>22.8 ± 6.8</td>
</tr>
</tbody>
</table>

* dATP and dGTP pools are the smallest dNTP pools in small thymocytes (p < 0.005).
* dCTP pool is the smallest dNTP pool in large thymocytes (p < 0.005).
* dTTP pool is the largest dNTP pool in large thymocytes (p < 0.005).

which are at the same phase of the cell cycle. Differences related to cell cycle are apparent when comparing dNTP levels of G_1 thymocytes and S phase thymocytes. In S phase thymocytes, dCTP is the smallest dNTP pool and dTTP is the largest pool. In contrast, dGTP and dATP are the smallest pools in G_1 thymocytes.

Kelford and Fox (31) recently reported somewhat lower levels of dNTP in thymocyte fractions separated by cell elutriation. The reasons for these differences are not clear, but it does not seem to be caused by nucleotide extraction or assay conditions since adaptation of their methods did not affect our results (data not shown). On the other hand, cell elutriation is a longer process and results in a much smaller enrichment of S thymocytes when compared to BSA gradient centrifugation (31). It is likely that the differences in dNTP levels arise from differences in cell composition and preparation procedures.

### Apparent Activities of Ribonucleotide Reduction and Deoxyribonucleoside Salvage in T Lymphocyte Populations—

Intracellular purine and pyrimidine dNTP can be synthesized by either ribonucleotide reduction or by deoxyribonucleoside salvage pathways. In order to determine the relative contribution of these alternative pathways in maintaining the vastly different intracellular dNTP pools among the T lymphocyte populations, we have compared the activities of individual deoxyribonucleotide biosynthetic pathways in these three T lymphocyte populations.

S phase thymocytes have significant ribonucleotide diphosphate reductase reaction activity (2 pmol/h/10^6 cells), whereas both G_1 thymocytes and peripheral blood T lymphocytes have no detectable activity (<0.1 pmol/h/10^6 cells).

Since both G_1 thymocytes and peripheral blood T lymphocytes lack de novo deoxyribonucleoside biosynthetic activity, any contribution to their dNTP pools could come either from deoxyribonucleoside salvage or alternatively be carried over from the previous S phase. In fact, there is good correlation between deoxyribonucleoside salvage activities and dNTP levels when G_1 thymocytes are compared to peripheral blood T lymphocytes. G_1 thymocytes have higher salvage activities of deoxyguanosine, deoxyctydine, and thymidine than do peripheral blood T lymphocytes (Table II). Deoxyadenosine is the only deoxyribonucleoside appreciably salvaged by peripheral blood T lymphocytes, and this corresponds to the relatively high dATP levels in these cells (Tables I and II). S phase thymocytes have higher salvage capabilities of all four deoxyribonucleosides when compared to either of the G_1 phase T lymphocyte populations.

### Regulation of Deoxyribonucleoside Salvage in G_1 and S Phase Thymocytes—

In order to elucidate possible regulatory mechanisms operating in the salvage of deoxyribonucleosides, G_1, and S phase thymocytes were incubated in the presence of an increasing concentration of either a single deoxyribonucleoside or a mixture of all four deoxyribonucleosides and the accumulation of the respective dNTPs was determined (Figs. 1 and 2). These experiments should elucidate both the salvage kinetics of the individual deoxyribonucleosides as well as any regulation of the salvage of one deoxyribonucleoside by the other three.

Incubation of S phase cells in the presence of increasing concentrations of extracellular deoxyadenosine results in up to 30-fold expansion of intracellular dATP pools (Fig. 1A). However, when S phase thymocytes are incubated in the presence of equimolar concentrations of all four deoxyribonucleosides, dATP accumulation from deoxyadenosine is markedly suppressed. Similar results are obtained when the incorporation of deoxyguanosine into intracellular dGTP pools of S phase thymocytes is measured (Fig. 1B), i.e., the incorporation of deoxyguanosine into the dGTP pool is suppressed in the presence of the other three deoxyribonucleosides. Deoxycytidine salvage, on the other hand, is limited even when S phase thymocytes are incubated with deoxycytidine alone and the addition of the other three deoxyribonucleosides does not further suppress deoxycytidine salvage (Fig. 1C). Thymidine salvage proceeds at a relatively high rate when S phase thymocytes are incubated with thymidine alone and dTTP accumulation is further enhanced in the presence of the other three deoxyribonucleosides (Fig. 1D). Thus, the dTTP pool is the only dNTP pool which markedly increases in the presence of increasing concentrations of all four deoxyribonucleosides (Fig. 1, A—D).

Qualitatively similar results are obtained when the salvage of deoxyribonucleosides in G_1 thymocytes is examined (Fig. 2). One significant difference is the absence of dTTP accumulation when G_1 thymocytes are incubated with thymidine alone (Fig. 2D). However, incubation of G_1 thymocytes in the presence of all four deoxyribonucleosides results in accumulation of dTTP as observed in S phase thymocytes.

### Table II

<table>
<thead>
<tr>
<th>Deoxyribonucleoside incorporated</th>
<th>Peripheral blood T lymphocytes</th>
<th>Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small (G_1 phase)</td>
<td>Large (S phase)</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>230</td>
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</tr>
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<td>Deoxyguanosine</td>
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<tr>
<td>Deoxyctydine</td>
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<td>128</td>
</tr>
<tr>
<td>Thymidine</td>
<td>3.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

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Deoxynucleotide Metabolism

Lymphocytes (1 × 10^6 cells) were incubated in 1 ml of RPMI 1640 containing 10% heat-inactivated FCS (36 °C, 30 min) in the presence of 10 μCi of the specified radioactive deoxyribonucleoside: [methyl-^3H]thymidine (77 Ci/mmol); [5^-3H]deoxycytidine (25 Ci/mmol); [8^-3H]deoxyguanosine (5 Ci/mmol); and [8^-3H]deoxyadenosine (15 Ci/mmol) in the presence of 10 μM 2′-deoxycoformycin. After 2-h incubation, nucleotides were extracted and purine and pyrimidine deoxyribonucleotides were separated by two-dimensional chromatography on polyethyleneimine-cellulose as described under "Experimental Procedures." Total incorporation was calculated from the sum of incorporation into the respective deoxyribonucleotides plus incorporation into DNA. The data are from one experiment; three other experiments gave similar results.
Deoxyribonucleotide Metabolism

The kinetics of dATP, dGTP, and dCTP accumulation in thymocytes incubated in the presence of all four deoxyribonucleosides is similar (Figs. 1 and 2). Since deoxycytidine, deoxyguanosine, and deoxyadenosine are phosphorylated by a single enzyme, deoxycytidine kinase (32), it is possible that the phosphorylation of all three deoxyribonucleosides is regulated at the level of phosphorylation. Interference with the phosphorylation of deoxyadenosine and deoxyguanosine by deoxycytidine could be due to either direct substrate competition or to feedback inhibition by dCTP (33). To test for possible interference by deoxycytidine in the phosphorylation of purine deoxyribonucleosides, thymocytes were incubated with increasing concentrations of deoxycytidine and their effect on the accumulation of dATP and dGTP from deoxyadenosine and deoxyguanosine, respectively, was determined (Fig. 3). The phosphorylation of both deoxyguanosine and

Fig. 1. Accumulation of intracellular deoxyribonucleoside triphosphates from extracellular deoxyribonucleosides by S phase thymocytes. S phase thymocytes (2 × 10⁶ cells/ml) were incubated overnight at 37 °C in RPMI 1640 with 10% FCS. After 16 h, deoxyribonucleosides were added and the cells were incubated for an additional 2 h. Nucleotides were extracted and deoxyribonucleoside triphosphates were determined as described under Experimental Procedures. ○, cells incubated in the presence of all four deoxyribonucleosides; ●, deoxyadenosine only; ●, deoxyadenosine and the three other deoxyribonucleosides. B, dGTP determinations: ○, deoxyguanosine only; ●, deoxyguanosine and the other three deoxyribonucleosides. C, dCTP determinations: ○, deoxycytidine only; ●, deoxycytidine and the other three deoxyribonucleosides. D, dTTP determinations: ○, thymidine only; ●, thymidine and the other three deoxyribonucleosides. 2'-Deoxycoformycin (10 μM) was added whenever deoxyadenosine was included. The data are from a single experiment done in duplicate; three additional experiments gave similar results.

Fig. 2. Accumulation of intracellular deoxyribonucleoside triphosphates by G1 phase thymocytes incubated in the presence of extracellular deoxyribonucleosides. G1 thymocytes were incubated with deoxyribonucleosides as described in Fig. 1. ○, cells incubated in the presence of a single deoxyribonucleoside; ●, cells incubated in the presence of all four deoxyribonucleosides at equimolar concentrations. A, dATP determinations: ○, deoxyadenosine only; ●, deoxyadenosine with the other three deoxyribonucleosides. B, dGTP determinations: ○, deoxyguanosine only; ●, with the other three deoxyribonucleosides. C, dCTP determinations: ○, deoxycytidine only; ●, deoxycytidine with the other three deoxyribonucleosides. D, dTTP determinations: ○, thymidine in the presence of the other three deoxyribonucleosides. 2'-Deoxycoformycin (10 μM) was added whenever deoxyadenosine was included. The data are from a single experiment done in duplicate; two additional experiments gave similar results.

Fig. 3. Effect of deoxycytidine on the accumulation of intracellular dATP and dGTP from deoxyadenosine and deoxyguanosine by G1 thymocytes. Incubation conditions were as described in Fig. 1. ○, dATP accumulation by G1 thymocytes incubated with 100 μM deoxyadenosine in the presence of the indicated deoxycytidine concentration. ○, dGTP accumulation by G1 thymocytes incubated with 100 μM deoxyguanosine in the presence of the indicated deoxycytidine concentration. Deoxyribonucleoside triphosphates were determined as described under Experimental Procedures. The data represent a single experiment done in duplicate; three additional experiments gave similar results.
deoxycytidine, present at high concentration (100 μM), is completely inhibited in the presence of micromolar concentrations of deoxyadenosine. In parallel, the phosphorylation of deoxycytidine to yield dCTP levels off at micromolar concentrations (Figs. 1 and 2). Since the $K_m$ for deoxycytidine phosphorylation by deoxycytidine kinase is 2 μM as compared to 180 and 300 μM for deoxyguanosine and deoxyadenosine, respectively (32), it is possible that deoxycytidine directly competes with deoxyguanosine and deoxyadenosine. However, it is also possible that the accumulated dCTP participates in the regulation of deoxycytidine, deoxyguanosine, and deoxyadenosine phosphorylation by exerting feedback inhibition of deoxycytidine kinase (33).

dTTP accumulation is enhanced in the presence of all four deoxynucleosides when compared to its accumulation in the presence of thymidine alone (Fig. 1). Since this effect is also observed in G1 thymocytes (Fig. 2), it cannot be explained on the basis of positive regulation of ribonucleotide reductase. Thymidine nucleotides can be synthesized from deoxycytidine following deamination either by cytidine deaminase or by dCMP deaminase (34); it is therefore possible that dTTP is formed also from deoxycytidine. To determine the contribution of deoxycytidine to dTTP accumulation, G1 thymocytes were incubated in the presence of increasing concentrations of deoxycytidine, and intracellular dTTP pools were measured (Fig. 4). Indeed, deoxycytidine contributes to dTTP accumulation, i.e. dTTP pools increase with increasing extracellular concentrations of deoxycytidine. Inhibition of cytidine deaminase by tetrahydrouridine (0.1 mM) does not affect dTTP formation from deoxycytidine (data not shown), suggesting the involvement of dCMP deaminase rather than deoxycytidine deaminase in the synthesis of dTTP from deoxycytidine.

**Fig. 4.** Accumulation of intracellular dCTP and dTTP by G1 phase thymocytes incubated in the presence of deoxycytidine. Incubation conditions were as described in Fig. 1. O, dCTP accumulation; O, dTTP accumulation. The data represent a single experiment done in duplicate; two additional experiments gave similar results.

**Regulation of Ribonucleotide Reduction in S Phase Thymocytes—**While G1 thymocytes can form dNTP only by deoxynucleoside salvage, ribonucleotide reduction may be a major contributor to dNTP pools in S phase large thymocytes (see above). Ribonucleotide diphosphate reductase, the enzyme which catalyzes the reduction of ribonucleotides, is tightly regulated by dNTP (18, 19). Salvage of extracellular deoxynucleosides in S phase thymocytes may result in accumulation of intracellular dNPT and may thereby affect the reduction of ribonucleotides. To investigate such a possible regulatory role, S phase thymocytes were incubated with each of the four deoxynucleosides to allow accumulation of the respective dNTP and their effect on the levels of the other three ribonucleotides was determined (Fig. 5). The results of these experiments were then analyzed in the context of the model of the regulation of ribonucleotide diphosphate reductase (18, 19).

**Fig. 5.** Effect of deoxynucleoside salvage on ribonucleotide reduction by S phase thymocytes. Incubation conditions were as described in Fig. 1. A, dATP determinations; S phase cells were incubated in the presence of the indicated concentration of deoxycytidine (O), thymidine (△), and deoxycytidine (□). B, dGTP determinations, incubation in the presence of thymidine (△), deoxycytidine (□), and deoxyguanosine (□). C, dCTP determinations; incubation in the presence of deoxyadenosine (□), thymidine (△), and deoxyguanosine (□). D, dTTP determinations; deoxycytidine (□), deoxyadenosine (□), and deoxyguanosine (□). The data represent a single experiment done in duplicate; two additional experiments gave similar results.

Effects of increasing concentrations of extracellular deoxycytidine, deoxyguanosine, and thymidine on intracellular dCTP levels in S phase-enriched thymocytes are illustrated in Fig. 5. Both thymidine and deoxyguanosine result in a 50–70% depletion of dCTP levels, consistent with the inhibition of CDP reduction by the respective accumulation of dGTP and dTTP (Figs. 1 and 5C). The S phase-enriched thymocyte fraction contains up to 40% G1 phase thymocytes which have no significant ribonucleotide reductase activity (data not shown). It is therefore likely that the actual inhibition of CDP reduction in S phase thymocytes is higher than 50–70%. In contrast, deoxyadenosine did not cause a significant decrease in dCTP levels, despite the accumulation of large amounts of dATP, a feedback inhibitor of ribonucleotide diphosphate reductase (Fig. 5C). The failure of dATP to inhibit CDP reduction has been also observed in other mammalian cells (35, 36). Extracellular thymidine caused an increase in dATP levels (Fig. 5E) and deoxyguanosine caused an increase in dATP levels (Fig. 5A), consistent with stimulation of UDP reduction by dTTP and ADP and CDP reduction by dGTP, as predicted from the model of ribonucleotide diphosphate reductase regulation (18, 19). Also consistent with this model is the increase in dATP levels in the presence of extracellular thymidine (Fig. 5A). However, the failure of deoxyguanosine and deoxyadenosine to deplete dTTP levels (Fig. 5D) is inconsistent with the model which predicts the inhibition of...
UDP and CDP reduction by the purine dNTP. Deoxycytidine, on the other hand, caused a significant increase in dTTP levels (Fig. 5D). This increase, however, is likely the result of deoxycytidine deamination and subsequent methylation yielding thymidine nucleotides (34) as indicated by similar results in G1 cells in the absence of ribonucleotide reduction activity (Figs. 2 and 4).

**DISCUSSION**

Maintenance of balanced dNTP levels during the S phase of the cell cycle is of the utmost importance to the fidelity of DNA synthesis (37). Alterations in intracellular dNTP pools result in increased rates of spontaneous mutations (22, 23). Severe imbalances in intracellular dNTP pools ultimately result in inhibition of DNA synthesis and cause cell death (18, 19, 38). Deoxynucleobase synthesis has been studied in cultured mammalian cells where, in the absence of extracellular deoxynucleobases, the only pathway leading to dNTP synthesis is ribonucleotide reduction (18, 19, 38). Thelander and Reichard (18) proposed a model explaining the regulation of ribonucleotide reduction maintaining balanced dNTP pools in the absence of deoxynucleobase salvage. However, in tissues with active deoxynucleobase salvage pathways, such as the thymus (15, 17, 31), it is likely that the salvage of extracellular deoxynucleobases also contributes to dNTP synthesis. Therefore, any model of dNTP synthesis in thymocytes should include the interaction between ribonucleotide reduction and deoxynucleobase salvage in maintaining balanced intracellular dNTP pools.

We propose a model (Fig. 6) that explains the possible interaction between ribonucleotide reduction and deoxynucleobase salvage in S phase thymocytes. In this model, ribonucleotide reduction is restricted mainly to the synthesis of purine dNTP while pyrimidine dNTPs are synthesized primarily by extracellular deoxynucleobase salvage. This model is based on the experimental results as outlined below. Deoxycytidine is efficiently phosphorylated in S phase thymocytes by deoxycytidine kinase which is active in all thymocyte populations (15, 17). Part of the dCMP formed by deoxycytidine kinase is further phosphorylated to dCTP while another part is deaminated to dUMP leading to dTTP synthesis (Figs. 1 and 4). The intracellular level of dCTP is tightly regulated by feedback inhibition of deoxycytidine kinase by dCTP (Figs. 1–3 and Ref. 33). Since deoxyguanosine and deoxyadenosine are also phosphorylated by deoxycytidine kinase, their salvage is also inhibited by dCTP; therefore, the intracellular dCTP pool should be smaller when compared to the other dNTP pools. Indeed, as predicted, dCTP is the smallest dNTP pool in S phase thymocytes (Table I). This result is in contrast to dNTP pools found in cultured cells where the dGTP pool is usually the smallest pool and dCTP is often the largest (18). However, these determinations were done under conditions that restrict deoxynucleobase salvage, i.e., in cultured cells in the absence of extracellular deoxynucleobases. In addition, dCTP expansion in cell lines may result from mutations in dCTP-metabolizing enzymes which are relatively frequent (21–23).

Studies correlating dNTP levels with DNA synthetic rates suggested that dCTP may be involved in the regulation of DNA synthesis (40). Since dCTP is the smallest dNTP pool in S phase thymocytes, regulation of the dCTP pool is important in the control of DNA synthesis. The role of dCTP could be especially significant in immunodeficiencies associated with adenosine deaminase or purine nucleoside phosphorylase deficiencies (1, 2). Lymphocytes in these patients accumulate large amounts of deoxyadenosine or deoxyguanosine, the substrates of the respective enzymes, which, after phosphorylation yield dATP and dGTP which subsequently inhibit CDP reduction (3–10, 18). In the case of S phase thymocytes, it is possible that adenosine deaminase and purine nucleoside phosphorylase deficiencies result in an unusually high intrathymic accumulation of deoxyadenosine and deoxyguanosine, respectively, which may compete with deoxycytidine for phosphorylation by deoxycytidine kinase, thereby inhibiting dCTP synthesis by both the salvage and ribonucleotide reduction pathways.

The model of dNTP regulation described above is based on the assumption that extracellular deoxynucleobase concentrations are between 1 and 100 μM. Measurements of serum deoxynucleobases have shown that pyrimidine deoxynucleobase concentrations are around 1 μM (41).
Since it is difficult to get extracellular thymic fluid, there are no data concerning extracellular deoxyribonucleoside concentrations in the thymus in vivo. It has been suggested that the majority of thymocytes die within the thymus, resulting in massive DNA degradation (42). As a result, it is likely that the concentrations of extracellular deoxyribonucleosides, the products of DNA degradation, may be higher in the intrathymic environment than in serum. If this is the case, then the proposed model for the interaction of ribonucleotide reduction with deoxyribonucleoside salvage may very well operate in the regulation of dNPT levels in S phase thymocytes in vivo.

Measurements of purine dNTP levels in G1 thymocytes and peripheral blood T lymphocytes allow comparison of T cells at different differentiation stages but in the same phase of the cell cycle (Fig. 1). G1 thymocytes have up to 100-fold higher levels of dNTP pools than peripheral blood T lymphocytes. No such differences are found in ribonucleotide pools. In fact, purine ribonucleotide pools are higher in peripheral blood T lymphocytes than in intrathymic T lymphocytes (data not shown). Higher deoxyribonucleoside salvage (Table II) capability combined with the lack of ribonucleotide reduction activity of G1 thymocytes (Table II) may indicate a major role for deoxyribonucleoside salvage in maintaining high levels of intracellular dNTP in G1 thymocytes (Table I).

The metabolic role of the relatively high dNTP observed in G1 thymocytes is unclear. DNA synthesis, the major consumer of dNTP, does not occur in small thymocytes which are at the G1 phase of the cell cycle. However, it may be significant that intrathymic lymphocytes are the only cells expressing appreciable activity of terminal deoxyribonucleotidyltransferase, a cell cycle-independent enzyme which uses dNTPs as substrates (43).

Acknowledgments—We wish to thank Ellen Thompson, Jacob Lee, and Changee Leung for expert technical assistance.

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