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Assessment of salvage pathways utilized for incorporation of exogenous pyrimidine nucleosides into DNA of guinea pig lymphocytes stimulated by Con A

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The organization of specific pyrimidine pathways to channel various nucleoside precursors into DNA is poorly understood. We show that concanavalin A-stimulated guinea pig lymphocytes incorporate [^{3}H]dThd, [^{3}H]dCyd, [^{3}H]dUrd, [^{3}H]Cyd and [^{3}H]Urd into DNA-thymines and DNA-cytosines in a highly conserved distribution pattern. DNA-thymines were labeled only by dThd and dUrd, while DNA-cytosines were labeled only by dCyd, Cyd and Urd. The kinetics for the incorporation of the [^{3}H]nucleosides were essentially identical, indicating equivalent abilities to measure DNA synthesis. Pyrazofurin inhibition of the pyrimidine de novo synthetic pathway inhibited cell proliferation and the levels of [^{3}H]nucleoside incorporation by approx. 50%, but did not alter restricted distribution of the [^{3}H]nucleosides among DNA-thymines and DNA-cytosines. These findings indicate the absence of Cyd and dCMP deaminase salvage pathways and suggest either subcellular compartmentalization or differential regulation of ribonucleoside diphosphoreductase which permits reduction of CDP but not UDP.

DNA synthesis; Pyrimidine salvage pathway; Pyrimidine de novo synthesis; Metabolic channeling; Pyrazofurin; Concanavalin A-stimulated T-lymphocyte

1. INTRODUCTION

Manipulation of lymphocyte proliferation with exogenous nucleosides and nucleoside analogs has proven useful in elucidation of nucleoside enzyme deficiencies associated with certain forms of inherited immunodeficiency disease [1,2] and the development of anti-cancer agents for use in lymphoid malignancies [3]. In order to further utilize various nucleosides and their interrelationships with lymphocyte cytotoxicity and cellular metabolism, an evaluation is needed of nucleoside metabolism and function in the context of subcellular compartmentalization and processing by multi-enzyme complexes [4]. Separate subcellular compartments of deoxynucleoside triphosphate pools for DNA synthesis [5–7] and multi-enzyme complexes capable of channeling dNTP precursors during synthesis without free access to intracellular pools [8,9] have been shown to exist. However, little is known about the compartmentalization of enzymes and metabolites involved in de novo and salvage pathway synthesis of deoxyribonucleotides.

Numerous studies have monitored the incorporation of various radioactive metabolites into lymphocyte DNA by monitoring acid insoluble fractions of cell lysates. Many of these studies have relied on the incorporation of the salvage pathway precursor, [^{3}H]dThd, as a specific marker for DNA synthesis. Somewhat offsetting the convenience of using [^{3}H]dThd to measure DNA synthesis is the awareness that the extent or even the

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Abbreviations: Con A, concanavalin A; HPhC, high-performance liquid chromatography

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absence of [^{3}H]dThd incorporation [5,8,10,11] may not accurately reflect the amount of DNA synthesis in a particular cell type. The use of other radiolabeled pyrimidine precursors from either the de novo or salvage pathway as alternative indicators of DNA synthesis share the complexities of possibly labeling RNA and DNA and being distributed among DNA-Thy as well as DNA-Cyt.

In the following studies we used HPLC analysis of isolated DNA hydrolysates to examine the ability of various radiolabeled nucleosides to label DNA-Thy and DNA-Cyt in concanavalin A-induced proliferation of guinea pig T-lymphocytes. These labeling patterns provide information about the various pyrimidine salvage pathways which are involved in supporting DNA synthesis, and are intended to generate information about subcellular compartmentalization of DNA precursors and interrelationships between these pathways and exogenously supplied nucleosides.

2. MATERIALS AND METHODS

2.1. *DNA labeling and isolation*

Lymphocytes were isolated from axillary, popliteal and inguinal lymph nodes of male Hartley strain guinea pigs (Biolab Corporation, St Paul, MN) that had been sensitized 7 days earlier with Freund's complete adjuvant (Difco, Detroit, MI). Lymphocytes were teased into suspension in Eagles' minimum essential medium (Gibco, Grand Island, NY) and filtered once through a stainless steel screen. Cells were washed twice by centrifugation at 600 × *g* and were seeded at 1×10^6 cells/ml in medium which contained 10 μg/ml of Con A (Sigma, St Louis, MO). Increases in cell number were routinely estimated as the increase in total cellular protein as measured by the method of Lowry et al. [12]. Lymphocyte DNA was labeled by adding to the culture medium 1 μCi/ml of [*methyl*-^{3}H]dThd (6.7 Ci/mM), [6-^{3}H]dUrd (21.4 Ci/mM), [5-^{3}H]dCyd (25.6 Ci/mM), [G-^{3}H]Urd (6.0 Ci/mM), [5-^{3}H]Cyd (21.0 Ci/mM), or [6-^{14}C]-orotic acid (51.3 mCi/mM) from New England Nuclear (Boston, MA). All radiolabeled compounds were checked for purity by HPLC analysis prior to use. Labeling of cells (25×10^6/data point) was initiated at 22 h after Con A stimulation and terminated at 52 h for samples used to determine radioactivity distributions among DNA bases. Shorter periods, i.e., 3 to 12 h, of culture in the presence of radioactive precursors were used in studies to determine relative rates of uptake into DNA. The incorporation for precursors was essentially linear through 22 h, slowed at 22–30 h, and leveled by 40–48 h. All culture periods were terminated by decanting the culture medium and immediately adding 25 ml of a solution containing 0.15 M NaCl, 0.1 M EDTA, 20 mg/ml of SDS, and nuclei from 40 g of rat liver. This mixture was heated 10 min at 60°C, and DNA was isolated as described [13]. DNA concentrations were based on a colorimetric assay following the method of Burton [14]. In experiments using pyrazofurin, 10 μM pyrazofurin was added at the beginning of culture along with Con A and was present throughout the experiment. For experiments in which nucleosides were added to reverse pyrazofurin inhibition, 75 μM nucleoside was added with pyrazofurin and was present throughout the culture period.

2.2. *HPLC analysis of DNA radiolabeling*

To determine which DNA bases were labeled by a particular radioprecursor, isolated DNA was hydrolyzed to purine and pyrimidine bases in 98% formic acid and the bases separated by HPLC on a Supelcosil LC-18-DB column (Supelco, Bellefonte, PA) using isocratic elution with a buffer containing 5 mM KH_2PO_4 (pH 4.0) and 0.1% tetrahydrofuran [15]. A typical analysis consisted of injecting 2000–8000 cpm of DNA hydrolysate, collecting 0.5 ml fractions and determining radioactivity content by liquid scintillation counting. Recovery of injected radioactivity was 94–98%.

3. RESULTS

3.1. *Labeling of DNA-Thy and DNA-Cyt by [^{3}H]nucleosides*

We first determined the distribution of various radiolabeled nucleoside precursors among DNA-pyrimidines of guinea pig lymphocytes replicating in response to Con A stimulation. Shown in fig.1 are ^{3}H-labeling distributions for the incorporation of the salvage pathway precursors, [^{3}H]dThd, [^{3}H]dCyd, [^{3}H]dUrd, [^{3}H]Cyd, [^{3}H]Urd, and the de novo pathway pyrimidine precursor, [^{14}C]orotic acid. While the de novo pathway precursor labeled

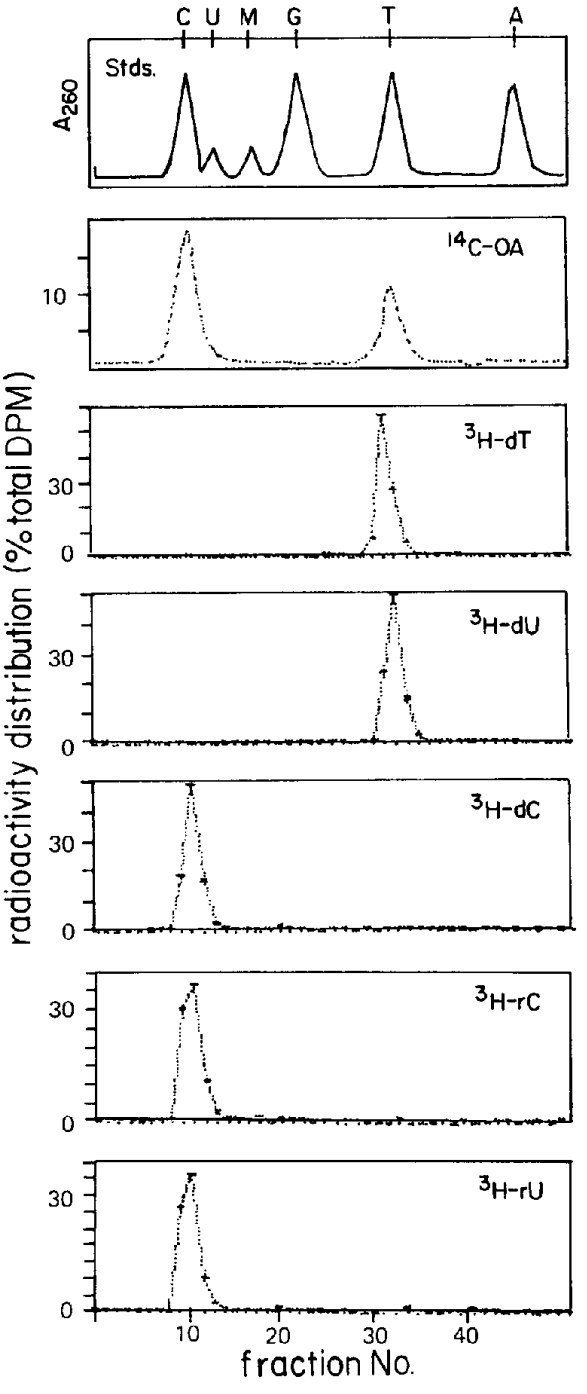

Fig.1. Distribution of radioactivity among DNA bases as labeled by various radioisotopic DNA precursors during Con A-stimulated lymphocyte proliferation. DNA was isolated from cultured lymphocytes, hydrolyzed then chromatographed as described in section 2. The top panel depicts the absorbance profile for HPLC separation of nucleic acid bases. C, cytosine; M, 5-methylcytosine; U, uracil; G, guanine; T, thymine; A, adenine; OA, orotic acid; dT, dThd; dU, dUrd; dC, dCyd; rC, Cyd; rU, Urd.

both DNA-Thy and DNA-Cyt, the salvage pathway precursors were greatly restricted with respect to their labeling distributions, i.e., [^{3}H]dThd and [^{3}H]dUrd labeled only DNA-Thy; [^{3}H]dCyd, [^{3}H]Cyd and [^{3}H]Urd labeled only DNA-Cyt.

The labeling of DNA-Thy and DNA-Cyt was further characterized by determining rates of precursor incorporation with [^{3}H]dThd or [^{3}H]dUrd and [^{3}H]dCyd or [^{3}H]Urd, respectively, as shown in fig.2. All four precursors were incorporated linearly through 12 h and demonstrated essentially identical rates though different extents of labeling, i.e., [^{3}H]dThd and [^{3}H]dCyd, 200–360 dpm/nmol pyrimidine; [^{3}H]dUrd and [^{3}H]Urd, 22–44 dpm/nmol pyrimidine. Thus, while the fate of a specific [^{3}H]nucleoside as a DNA-Thy or DNA-Cyt was restricted, the corresponding overall measures of DNA synthesis were the same for the nucleosides.

3.2. *Nucleoside incorporation during pyrazofurin-blockade of the de novo pathway*

Pyrazofurin is an effective inhibitor of Con A-stimulated lymphocyte proliferation via blockage of DNA synthesis [16]. It acts through inhibition of orotidylate decarboxylase and the subsequent supply of de novo pathway pyrimidine nucleotides for DNA synthesis [17,18]. We used pyrazofurin to determine (i) the relative contribution of pyrimidine deoxynucleoside triphosphate from the de novo versus salvage pathways for use in lymphocyte proliferation, and (ii) to determine if inhibition of the de novo pathway could alter the DNA-labeling distribution of the [^{3}H]nucleosides. Pyrazofurin (10 μM) was added at time zero along with Con A and was present throughout the culture period. The results in fig.3a show that while 10 μM pyrazofurin reduced the de novo precursor [^{14}C]orotic acid incorporation by >90%, its inhibition of the salvage pathway [^{3}H]nucleosides was generally only 40–50%. This paralleled a 50% inhibition of cell proliferation

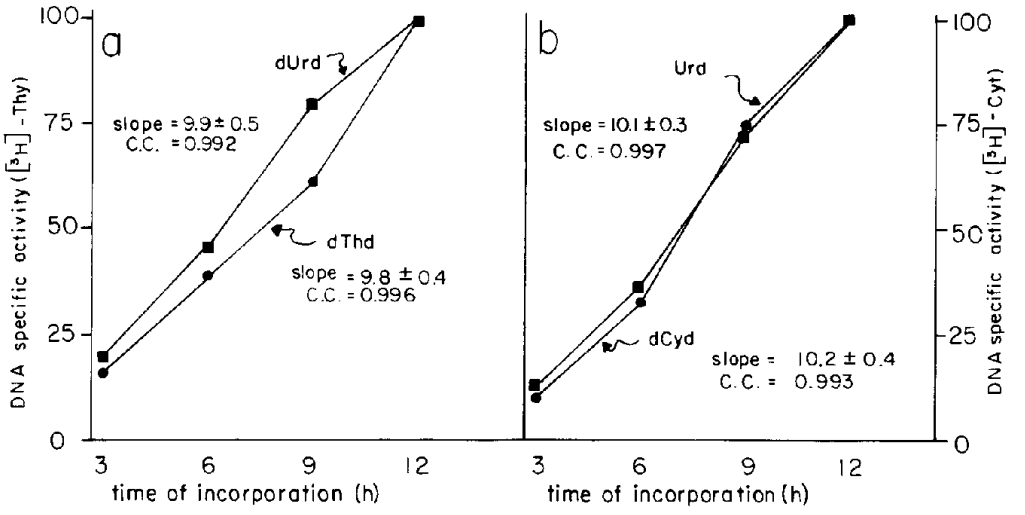

Fig.2. Rates of DNA synthesis by DNA-Thy and DNA-Cyt specific precursors. ^{3}H-labeled nucleosides were incorporated over a 12-h period and DNAs were isolated and analyzed for the radioactivity content of their HPLC Thy or Cyt peaks as described in section 2. Individual points represent the mean of 3 experiments and the slope values are the mean ± SE of the slopes from 3 experiments. (a) DNA-Thy precursors; (b) DNA-Cyt precursors.

(fig.3b). When the labeled DNAs from pyrazofurin-inhibited cultures were hydrolyzed and analyzed by HPLC for changes in the distribution of label among DNA-Thy and DNA-Cyt, the patterns were identical to those in fig.1, i.e., no new patterns of distribution appeared.

The reversal of pyrazofurin inhibited DNA synthesis and cell proliferation by exogenously supplied Urd has previously been reported for Con A-stimulated rat lymphocytes [16]. A similar reversal of 10 μM pyrazofurin-inhibited incorporation of [^{3}H]dThd into DNA and lymphocyte cell proliferation was observed when 75 μM Urd was added simultaneously with pyrazofurin to the cultures (fig.3b). None of the other nucleosides studied, i.e. dThd, dCyd, dUrd or Cyd, were able to reverse pyrazofurin inhibition.

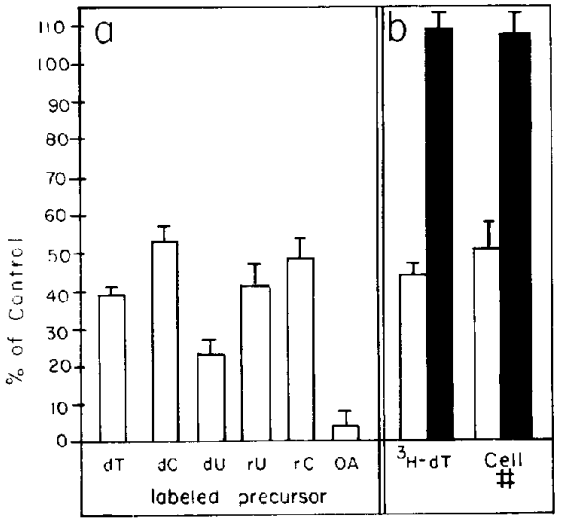

Fig.3. Pyrazofurin inhibition of DNA synthesis and cell proliferation. (a) Various radiolabeled DNA precursors were incorporated into Con A-stimulated lymphocytes in the absence or presence of 10 μM pyrazofurin and the level of labeling in isolated DNA determined as described in section 2. (b) Reversal of pyrazofurin-inhibited DNA synthesis ([^{3}H]dThd incorporation) and cell proliferation (cell no.) by the presence of 75 μM Urd during culture. All data are reported as % untreated controls and represent means ± SE for 3 or more determinations. dT, dThd; dC, dCyd; dU, dUrd; rU, Urd; rC, Cyd; OA, orotic acid; open bars, 10 μM pyrazofurin; solid bars, 10 μM pyrazofurin and 75 μM Urd.

4. DISCUSSION

The data presented in this communication establish that [^{3}H]dThd and [^{3}H]dUrd exclusively labeled DNA-Thy, while [^{3}H]dCyd, [^{3}H]Urd and [^{3}H]Cyd exclusively labeled DNA-Cyt during DNA synthesis in the Con A-stimulated proliferation of T-lymphocytes from guinea pig lymph nodes. Furthermore, the labeling pattern of salvage pathway nucleosides is not altered when de novo biosynthesis of pyrimidines is blocked by pyrazofurin. Con A stimulates proliferation primarily among T-cell lymphocytes [19]; the accompanying DNA synthesis has been characterized as semiconservative DNA replication with no appreciable evidence for repair synthesis or selective amplification of certain portions of the genome [20]. As illustrated in fig.4, entry of [^{3}H]dThd and [^{3}H]dCyd into DNA is mediated primarily by phosphorylation reactions of the salvage pathway, while entry of dUrd, Urd and Cyd also requires the action of thymidylate synthetase, CTP synthetase and ribonucleoside diphosphate reductase, respectively. The inability of [^{3}H]Cyd to label DNA-Thy confirms a previous report [16] of the absence of Cyd deaminase activity in Con A-stimulated T-lymphocytes, and also suggests the absence of dCMP deaminase activity. An explanation for restricted incorporation of [^{3}H]Urd into just DNA-Cyt would appear to be more complex. While [^{3}H]Urd must utilize ribonucleoside diphosphoreductase as it goes from CDP to dCDP for the labeling of DNA-Cyt, it is not reduced by ribonucleoside diphosphoreductase in the step UDP to dUDP which would allow it to be used as a precursor for labeling DNA-Thy. Information concerning the differential regulation of the reduction of ribonucleotides [21] does not readily account for the selective reduction of Urd to dCDP but not to dUDP. Furthermore, since [^{14}C]orotic acid labeled both DNA-Thy and DNA-Cyt, it must be presumed that either a dCMP deaminase activity (dCMP to dUMP to dTMP to dTTP) or UDP reductase activity (UDP to dUDP to dUMP to dTMP to dTTP) exist intracellularly in order for orotic acid to label DNA-Thy. A model of compartmentalization of dNTP pools through precursor channeling can account for the above incorporation patterns. This model would require separate de novo and salvage pathway UMP compartments, one of which would be channeled by a de novo multi-enzyme complex with tight coupling between UMP synthetase and enzymes needed to synthesize both dTTP and dCTP, including ribonucleotide reductase, cytidine triphosphate synthetase and thymidylate synthetase. A second, salvage pathway complex not tightly coupled to UMP synthetase and able to channel Urd only to dCTP would be required to contain ribonucleotide reductase and cytidine triphosphate synthetase activities but not thymidylate synthetase activity. Evidence for the existence of a 'replitase' complex comprised of ribonucleoside reductase, thymidylate synthetase, topoisomerase, and DNA polymerase α in HeLa cells has been reported [9]. The existence of a similar multi-enzyme complex containing CTP synthetase and ribonucleoside reductase have not been reported, but would be predicted to exist in Con A-stimulated guinea pig lymphocytes. A similar analysis of the ability of various DNA precursors to selectively label DNA-Thy and DNA-Cyt of other proliferating cells may

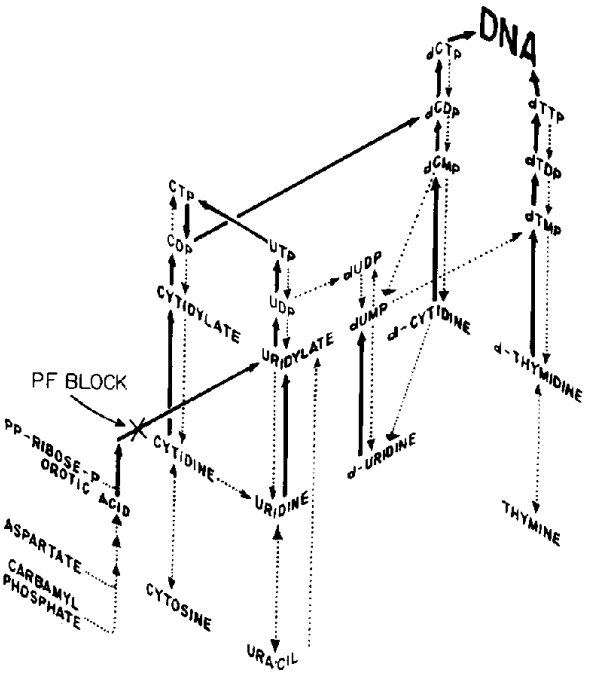

Fig.4. Summary of pyrimidine pathways in eukaryotic cells. Solid line arrows indicate routes utilized by Con A-stimulated guinea pig lymphocytes to incorporate various exogenous nucleosides into DNA.

prove helpful in establishing an understanding of nucleoside metabolism as it relates to subcellular compartmentalization and precursor channeling by multi-enzyme complexes.

The ability of exogenous Urd to reverse pyrazofurin inhibition while only being incorporated in DNA-Cyt does not support as a mechanism for its action the salvage pathway resupply of depleted dTTP and dCTP pools for DNA synthesis. Instead, it would appear that Urd may act at a level which directly interferes with the ability of pyrazofurin to exert its inhibitory influence on orotidylate decarboxylase. The ability of only Urd among the nucleotides tested to reverse pyrazofurin inhibition may be related to recent observations of selective and concentrative Urd uptake by murine splenocytes [22]. Selective transport of exogenous Urd by Con A-stimulated lymphocytes may provide an intracellular concentration of Urd sufficient to block pyrazofurin inhibition. Additional studies to establish the precise mechanisms for Urd reversal of pyrazofurin inhibition are in progress.

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