# Uridine as the only alternative to pyrimidine de novo synthesis in rat T lymphocytes

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Concanavalin A-induced proliferation of rat T-lymphocytes is completely inhibited by $10^{-5}$ M pyrazofurin, a potent inhibitor of pyrimidine de novo synthesis, as judged by cell viability and [$^3$H]thymidine incorporation. Proliferation is completely restored by $5 \times 10^{-5}$ M uridine. Cytidine, deoxycytidine, deoxyuridine and thymidine $10 \times 10^{-5}$ M each, fail to re-establish proliferation but produce an isotropic dilution of [$^3$H]thymidine uptake in DNA. Bases (cytosine, uracil and thymine) neither restore proliferation nor induce isotopic dilution. The unexpected inability of cytidine to reverse de novo pyrimidine synthesis inhibition suggests a lack of cytidine deaminase activity in rat T-lymphocytes. This is confirmed by a direct sensitive radioisotopic assay ($< 0.001$ nmol . min$^{-1}$ . $10^{-6}$ cells).

*Con A-stimulated T lymphocytes* *Pyrimidine de novo synthesis* *Pyrimidine salvage pathways* *Pyrazofurin* *Uridine* *Cytidine deaminase*

## 1. INTRODUCTION

Inhibition of pyrimidine de novo biosynthesis in different malignant cell systems by pyrazofurin (PF), a potent inhibitor of orotidylate decarboxylase [1,2], could be reversed by addition of exogenous nucleosides. In every case, not only uridine (Urd) was effective but also cytidine (Cyd) [1,2] or the combination of deoxycytidine (dCyd) and deoxyuridine (dUrd) [2].

Here, we used complete Freund's adjuvant (CFA)-activated lymph nodes as a source of T lymphocytes [3] to study the different metabolic pathways of pyrimidine nucleotide biosynthesis involved in these cells. As found in other systems, con A-activated T cells' proliferation was completely inhibited in the presence of $10^{-5}$ M PF. However, we found that only uridine could reverse such an inhibition. Furthermore, in the absence of PF, 3 nucleosides (cytidine, deoxycytidine and deoxyuridine) which did not reverse PF inhibition, were able to provide an important decrease of [$^3$H]deoxythymidine (d[$^3$H]Thd) incorporation into DNA, showing the importance of the different pyrimidine metabolism pathways in rat T lymphocytes.

## 2. MATERIALS

Chemicals used included: pyrazofurin (4-hydroxy-5-$\beta$-D-ribofuranosylpyrazole-3-carboxamide), a gift from Dr Davis (Lilly Res. Lab., Indianapolis IN); pyrimidine bases and nucleosides from Boehringer (Mannheim); Bacto complete Freund's adjuvant (CFA) from Difco Lab. (Detroit MI); [6-$^3$H]thymidine (1 Ci/mmol), [U-$^{14}$C]cytidine were purchased from CEA (Gifs/Yvette) and all other products as in [4].

## 3. METHODS

Three-months-old Sprague Dawley rats were injected twice in the hind footpads with CFA. Five days after the second injection, popliteal lymph nodes were aseptically removed and dissociated in the culture medium described below. After cell viability control, the cellular suspension was ad-

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justed to $10^6$ cells/ml in Dulbecco's medium modified according to [5] and supplemented with 33 mM Hepes, 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cell suspension with or without con A (5 μg/ml) was dispensed into 96-well microtiter plates (3040 Falcon Labware, Oxnard CA). PF ($10^{-5}$ M final) and effectors were added at culture initiation. Each culture contained $1.8 \times 10^5$ cells in 0.2 ml final vol. After 48 h incubation at 37°C in 5% $CO_2$, cultures were pulsed for 18 h with 1 μCi/well of d[$^3$H]Thd. d[$^3$H]Thd incorporation in DNA was then quantitated as in [6]. All assays were done in triplicate. Cultures were run in parallel to count the cells after the 3 days of culture.

Cytidine deaminase activity was determined by radioisotopic assay. The reaction mixtures contained $55 \times 10^{-3}$ M Tris–HCl (pH 7.4), $210 \times 10^{-6}$ M [U-$^{14}$C]cytidine and cell free extract in 200 μl final vol. The reaction was run at 37°C. At various times, 50 μl samples were taken and the reaction was stopped by heating at 96°C for 2 min. Cytidine, uracil and uridine were separated by descending chromatography on Whatman $CM_{82}$ paper in distilled water. Cytidine deaminase activity was calculated from the percentage of radioactivity appearing in uridine + uracil/min. The reaction was linear with time and cell-free extracts volume. The limit of detection of the method was 0.007 nmol/min. HPLC analysis of foetal calf serum (FCS) was performed as in [7].

## 4. RESULTS

### 4.1. *PF inhibition of con A-stimulated T lymphocytes proliferation*

Under our medium conditions, con A (5 μg/ml) induced a maximal lymph node T-cell proliferation after 3 days of culture. This was estimated by the total number of cells (blasts represented ~ 100%) and the incorporation of d[$^3$H]Thd in the acid-insoluble cell fraction. Such an activation was completely inhibited when PF was present at $10^{-5}$ M, which was in our system the optimal inhibitory concentration (not shown).

### 4.2. *Reversion of PF inhibition by pyrimidine bases and nucleosides (fig.1)*

As in [1,2], uridine was able to reverse the inhibitory action of PF. The blastogenesis was entirely restored by this nucleoside at a concentration of 50 μM. We tested also the capacity of pyrimidine bases (cytosine, uracil and thymine) and nucleosides (cytidine, deoxyuridine and deoxythymidine) to restore proliferation. No effect was observed with any of these compounds over 10–500 μM.

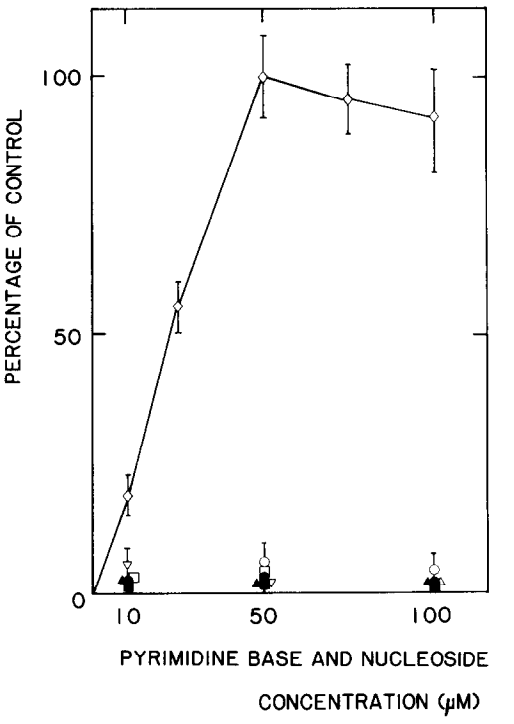


Fig.1. Incorporation of d[$^3$H]Thd into the acid-insoluble material of con A-stimulated rat T lymphocytes cultured in the presence of PF ($10^{-5}$ M) and pyrimidine bases and nucleosides: (○) uracil; (△) cytosine; (□) thymine; (◇) uridine; (▽) cytidine; (●) deoxyuridine; (▲) deoxycytidine; (■) thymidine. The conditions of culture, and the acid-insoluble material extraction are described in section 2. Each value is the mean of the results obtained in 4 separate experiments (the bars represent the SD). 'Control' represents the value of d[$^3$H]Thd incorporation in DNA in the presence of con A alone (130 000–200 000 cpm).

### 4.3. *Effect of pyrimidine bases and nucleosides on d[$^3$H]Thd incorporation in DNA (fig.2)*

Such a failure of pyrimidine bases and nucleosides (except uridine) to bypass PF inhibition of pyrimidine de novo biosynthesis apparently con-

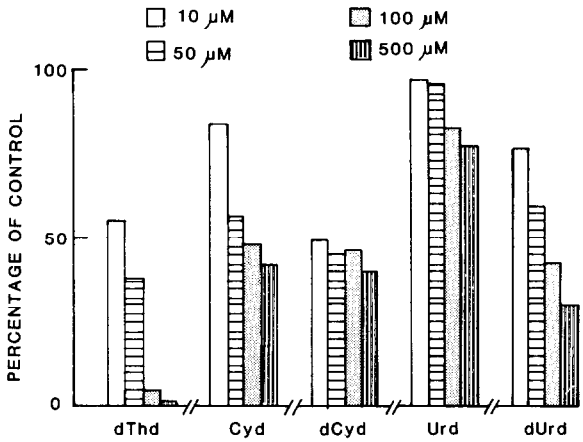


Fig.2. d[$^3$H]Thd incorporation into the acid-insoluble material of con A-stimulated rat T lymphocytes cultured in the presence of pyrimidine nucleosides. All assays were performed as in section 2 and without PF. Each value represents the mean of separate experiments (SD 15%). 'Control' represents the value of d[$^3$H]Thd incorporation into DNA in the presence of con A alone (130 000–200 000 cpm).

trasts with the effects of these compounds on d[$^3$H]Thd incorporation into DNA in the absence of PF. Thus dUrd, Cyd and dCyd gave a 50% decrease of d[$^3$H]Thd incorporation at $< 100\ \mu$M. Surprisingly, uridine was less effective, 500 $\mu$M giving only a 25% decrease. In no case was any modification of the total number of blasts found, indicating that the effects observed were indeed an inhibition of d[$^3$H]Thd incorporation in DNA and not a toxic effect. It should also be noted that uridine by itself did not produce any enhancement of the cell proliferation. This was tested over 10–500 $\mu$M (not shown).

### 4.4. *Determination of cytidine deaminase activity*

Cytidine deaminase activity in unstimulated and con A-stimulated rat T lymphocytes was $< 0.001$ nmol $\cdot$ min$^{-1} \cdot 10^{-6}$ cells (8 determinations). By contrast, an activity of 0.28 nmol $\cdot$ min$^{-1} \cdot 10^{-6}$ cells (mean of 5 determinations) was found in unstimulated human peripheral lymphocytes.

## 5. DISCUSSION

Under our culture conditions when de novo biosynthesis was inhibited, neither the medium we used (supplemented with 10% FCS which contains $\sim 5\ \mu$M of uridine as estimated by HPLC dosage), nor nucleic acids degradation (in this type of lymphocyte proliferation an important number of cells, principally B cells which are not con A-stimulated, die early) were sufficient to provide enough substrates to sustain an adequate salvage pathway. Therefore, we conclude that without any external addition, the con A-induced proliferation depends exclusively on the activity of pyrimidine de novo biosynthesis. Furthermore, when uridine is added to the medium, the absence of any increase in cell proliferation indicates that de novo pathway is able to provide a maximum response to mitogen. This is in contrast with the general assumption that most of the pyrimidine nucleotides are supplied through the salvage pathway in mitogen-stimulated lymphocytes [8] and is most consistent with the findings [9] of a prominent role for the de novo biosynthesis. Conversely, the use of PF allowed us to estimate the activity of uridine salvage pathway alone. In the two cases studied we did not observe differences in blastogenesis. Our results indicate that both metabolic pathways (de novo synthesis and salvage pathway) are equally effective in producing maximal T-cell proliferation.

The fact that among pyrimidine compounds, only uridine could reverse PF inhibition, permits an evaluation of the physiological importance of the different pyrimidine salvage pathways (fig.3). In our experiments, no effect was observed with cytidine, which contrast with the findings in other cell systems [1,2] where cytidine was as efficient as uridine to reverse PF inhibition. From that, it can be concluded that cytidine deaminase is not active in stimulated lymph-node rat T lymphocytes. This was confirmed by direct assay of enzymatic activity. Similarly, the lack of effect of uracil indicates that the cells cannot salvage uracil either by uracil–phosphoribosyltransferase or by uridine phosphorylase activities. Deoxycytidine and deoxyuridine did not restore cell proliferation, which confirms the incapacity of the cells to salvage the base moiety of these nucleosides, and indicates that a transfer between ribose and deoxyribose does not operate among pyrimidine nucleosides.

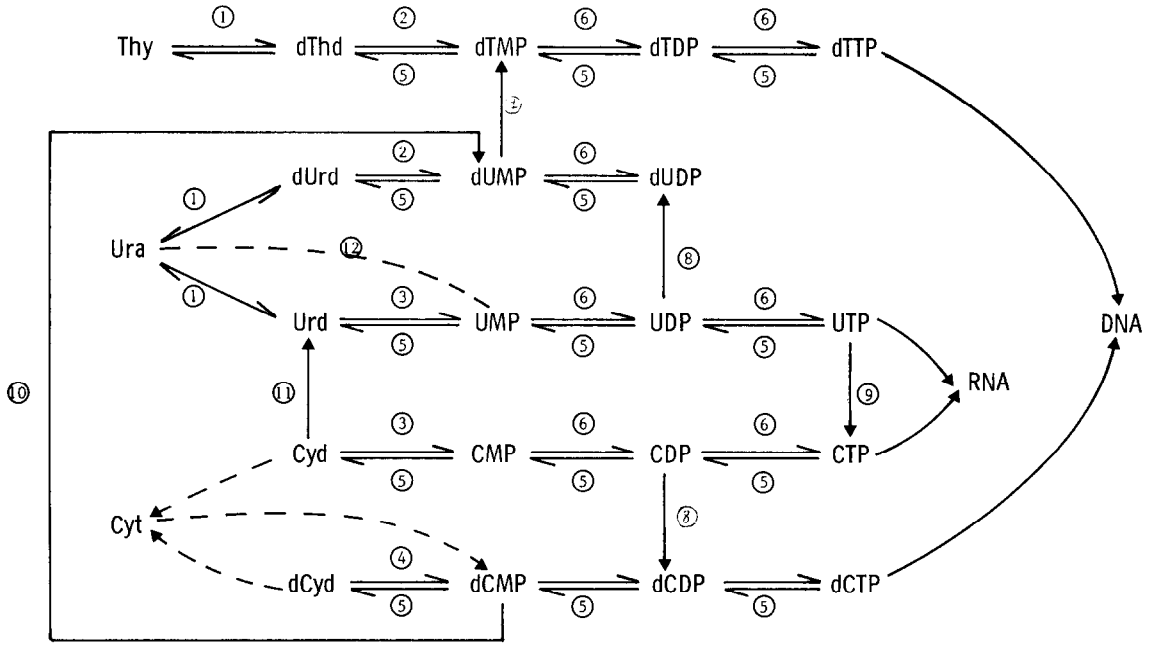


Fig.3. Pyrimidine salvage pathways in eukaryotic cells: (1) pyrimidine nucleoside phosphorylases – thymidine phosphorylase, EC 2.4.2.4; uridine phosphorylase, EC 2.4.2.3; (2) thymidine kinase, EC 2.7.1.21; (3) uridine–cytidine kinase, EC 2.7.1.48; (4) deoxycytidine kinase, EC 2.7.1.74; (5) 5′-pyrimidine nucleotidases – thymidylate 5′ phosphatase, EC 3.1.3.35; (6) nucleoside kinases – nucleoside monophosphate kinase, EC 2.7.4.14; thymidylate kinase, EC 2.7.4.9; nucleoside diphosphokinases, EC 2.7.4.6; (7) thymidylate synthase, EC 2.1.1.b; (8) ribonucleoside diphosphoreductase, EC 1.17.4.1; (9) cytidine triphosphate synthetase, EC 6.3.4.2; (10) deoxycytidine monophosphate deaminase, EC 3.5.4.12; (11) cytidine deaminase, EC 3.5.4.5; (12) orotate phosphoribosyltransferase, EC 2.4.2.10.

The inhibition by pyrimidine nucleosides of tritiated thymidine incorporation into DNA may be due to an interaction with thymidine transport through the plasma membrane, and/or with intracellular thymidine metabolism. Nucleoside transport in cultured cells is a controversial matter. From studies in various cell [10] it was concluded that thymidine, deoxycytidine and uridine are transported by a single carrier. In contrast, in phytohemagglutinin stimulated pig lymphocytes, the thymidine transport system was reported different from that for uridine and that for deoxycytidine [11,12]. If this was also the case for mitogen-stimulated rat lymphocytes, the effects we observed would be better explained by intracellular synthesis of thymidylate from non-radioactive nucleosides, and isotopic dilution of d[$^3$H]Thd in thymidine nucleotide pool(s), a mechanism similar to the deoxyuridine suppression of d[$^3$H]Thd incorporation in DNA in bone marrow cells [13]. This, and the results discussed above, suggest that cytidine and deoxycytidine if they cannot fulfil the pyrimidine requirements of the cells can however be salvaged by rat T lymphocytes by the sequential activity of the following enzymes:

*– for cytidine*

cytidine kinase → ribonucleoside diphosphate reductase → dCMP deaminase → thymidylate synthase

*– for deoxycytidine*

deoxycytidine kinase → dCMP deaminase → thymidylate synthase

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