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THE EFFECT OF EXTERNAL DEOXYRIBONUCLEOSIDES ON DEOXYRIBONUCLEOSIDE TRIPHOSPHATE CONCENTRATIONS IN HUMAN LYMPHOCYTES

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Abstract—The effects of deoxyribonucleosides on the intracellular levels of deoxyribonucleoside triphosphates (dNTP) and on the rate of labelled thymidine incorporated into DNA of human phytohaemagglutinin-stimulated lymphocytes have been studied. Thymidine (10^{-2}–10^{-6} M) expanded the dTTP and reduced dATP and dCTP levels. Deoxycytidine (10^{-3} M) expanded the dCTP level and caused inhibition of [^{3}H]thymidine incorporation into DNA but had no detectable effect on the other dNTP concentrations. Deoxyadenosine (10^{-3} M) expanded the dATP level, and reduced the other dNTP levels and deoxyguanosine (10^{-4} M) expanded the dGTP level and reduced the dCTP level; both inhibited [^{3}H]thymidine incorporation into DNA. The sensitivity of these cells to the addition of deoxynucleosides to their culture medium indicates that the plasma and tissue levels of nucleosides may profoundly influence DNA synthesis by human cells *in vivo*.

DNA synthesis of mammalian cells in tissue cultures may be inhibited by the addition of nucleosides to the external medium [1–3]. The growth of L5178Y and P815Y cells is inhibited by high concentrations of thymidine and this inhibition may be overcome if deoxycytidine is present [2]. In 3T6 cells, the growth inhibitory effect of adenosine may be prevented by uridine [3].

Deoxyguanosine causes chromosomal breakage in Ehrlich ascites cells, and these changes are prevented by deoxycytidine [4]. These cytotoxic effects of nucleosides have been ascribed to alterations in intracellular nucleotide concentrations, since nucleotides have been shown to be allosteric effectors of many enzymes involved in DNA synthesis [5–7] and catabolism [8, 9]. The degree of inhibition by individual nucleosides may vary in different species and tissues due to differences in the controls of different biochemical pathways. The present studies report the effects of external deoxyribonucleosides on the intracellular concentrations of deoxyribonucleoside triphosphates (dNTP) and rate of DNA synthesis of proliferating human cells. Phytohaemagglutinin-stimulated lymphocytes have been used as a model cell system.

MATERIALS AND METHODS

Reagent chemicals were supplied by British Drug Houses and Sigma Chemicals. Calf thymus DNA nucleosides and nucleotides were purchased from Sigma Chemicals, *Micrococcus luteus* DNA polymerase and poly d(A-T) from Miles Laboratories. [^{3}H]dATP (14 Ci/m-mole), [^{3}H]dTTP (23·7 Ci/m-mole), [^{3}H]thymidine (^{3}H-methyl) (5 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham, England. Medium TC 199 and phytohaemagglutinin (PHA) were purchased from Burroughs–Wellcome Limited.

Cell culture. The methods used for collecting and culturing human lymphocytes were those of Das and Hoffbrand [10]. Separated lymphocytes were incubated at 37° in 3-ml volumes at a cell concentration of 10^6/ml in medium TC 199 containing 30% autologous serum and 0·1 ml PHA. After 72 hr incubation cultures were pooled and, where appropriate, nucleosides or equal volumes of saline added. After incubation for 1 hr, the mixtures were rapidly cooled to 4°, collected by centrifugation at 4°, and washed once in ice-cold phosphate-buffered saline, pH 7·4. One ml of 60% methanol was added to the cell pellet for nucleotide extraction. The assay of deoxyribonucleoside triphosphates has been described [11]. Poly d(A–T) 0·05 units was used as template for assaying dTTP and dATP, and DNA (5 μg) as template for dCTP and dGTP in total assay volumes of 200 μl. [^{3}H]dATP was the labelled dNTP used in the assay of dTTP, dGTP and dCTP. [^{3}H]dTTP was used to assay dATP. All results are the mean of triplicate assays. Appropriate corrections were made for isotopic dilution [12]. Uptake of tritiated thymidine ([^{3}H]TdR) into DNA was also studied. Two μCi [^{3}H]TdR were added to each culture and incubated for 1 hr. DNA was extracted [13] and the radioactivity incorporated counted in a liquid scintillation counter.

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Table 1. The effect of thymidine (10^{-2} M–10^{-8} M) ± deoxycytidine (10^{-3} M–10^{-6} M) incubated for 1 hr on the dNTP levels of PHA-stimulated lymphocytes (72-hr cultures)

Experiment	Drug	Concn (M)	dATP (pmoles/10^6 cells)	dTTP (pmoles/10^6 cells)	dCTP (pmoles/10^6 cells)	dGTP (pmoles/10^6 cells)
I	Saline	--	5·0 (5·8)	17·0 (17·8)	3·2 (3·4)	1·9 (2·0)
	Saline	—	5·0 (5·8)	17·0 (17·8)	3·0 (3·1)	2·0 (2·1)
	Thymidine	10^{-2}	0 (0)	105 —	2·0 (2·0)	2·3 (2·3)
	Thymidine	10^{-3}	1·1 (2·0)	78 (78·9)	2·8 (2·8)	2·5 (2·5)
	Thymidine	10^{-4}	1·7 (2·5)	46 (46·8)	2·8 (2·8)	2·3 (2·3)
	Thymidine	10^{-5}	3·6 (4·9)	35 (36·2)	3·1 (3·2)	2·3 (2·4)
	Thymidine	10^{-6}	4·1 (5·0)	22·5 (23·4)	3·1 (3·2)	2·1 (2·2)
	Thymidine	10^{-7}	4·8 (5·7)	19 (19·9)	3·4 (3·6)	2·1 (2·2)
	Thymidine	10^{-8}	5·0 (5·7)	14 (14·7)	3·3 (3·4)	2·3 (2·4)
	Thymidine	10^{-4}	1·7 (2·9)	72·5 (73·7)	4·6 (4·7)	2·4 (2·4)
	+ deoxycytidine	10^{-3}				
	Thymidine	10^{-3}	1·8 (3·1)	71·0 (72·2)	4·8 (4·9)	2·4 (2·4)
	+ deoxycytidine	10^{-5}				
	Thymidine	10^{-3}	2·0 (3·3)	67 (68·3)	4·1 (4·2)	2·9 (3·0)
	+ deoxycytidine	10^{-6}				
II	Saline	--	4·4 (4·8)	8·2 (8·6)	2·4 (2·5)	1·9 (2·0)
	Saline	—	4·0 (4·3)	8·0 (8·3)	2·2 (2·2)	1·7 (1·8)
	Thymidine	5×10^{-3}	0 --	50 —	1·5 (1·5)	2·6 (2·6)
	Thymidine	10^{-4}	1·4 (1·7)	19·0 (19·2)	1·6 (1·6)	1·8 (1·8)
	Thymidine	10^{-5}	3·3 (3·7)	13·0 (13·4)	1·9 (2·0)	1·7 (1·8)
III	Saline		3·5 (3·7)	6·0 (6·2)	3·2 (3·3)	1·2 (1·2)
	Thymidine	10^{-2}	0 —	50 —	1·6 (1·6)	1·0 (1·0)
	Deoxycytidine	10^{-3}	2·7 (2·9)	6·2 (6·4)	7·6 (7·8)	2·5 (2·8)
	Thymidine	10^{-2}	0 --	50 —	4·5 (4·5)	3·3 (3·3)
	+deoxycytidine	10^{-3}				

Results are the mean of triplicate experiments.
Numbers in parentheses are corrected for isotope dilution [12].

RESULTS

The results are given in Tables 1–4, both corrected for isotope dilution in parentheses, and, the uncorrected data. Table 1 shows the effects of incubation of 72 hr normal PHA-stimulated lymphocytes for 1 hr with concentrations of thymidine ranging from 10^{-2} to 10^{-8} M on the cell levels of dNTP. At concentrations between 10^{-2} and 10^{-6} M thymidine expanded the dTTP and reduced both the dATP and dCTP levels. The fall in dATP was greater than that of dCTP at all concentrations of thymidine and was apparent at concentrations of thymidine of 10^{-5} M and 10^{-6} M which had no detectable effect on the dCTP level. Deoxycytidine (10^{-3}–10^{-6} M) prevented the fall in dCTP but not that of dATP caused by thymidine (10^{-3} M).

Table 2 shows the effects of different concentrations of deoxycytidine on the dNTP. Deoxycytidine, 10^{-3} M, markedly increased the level of dCTP and a less marked rise in dCTP occurred with external deoxycytidine concentrations of 10^{-4} M and 10^{-5} M. However, no significant change occurred in the other

Table 2. The effect of deoxycytidine (10^{-3} M–10^{-7} M) incubated for 1 hr on the dNTP levels and the incorporation into DNA of [^{3}H]thymidine by PHA-stimulated lymphocytes (72-hr cultures)

Experiment	Drug	Concn (M)	dATP (pmoles/10^6 cells)	dTTP (pmoles/10^6 cells)	dCTP (pmoles/10^6 cells)	dGTP (pmoles/10^6 cells)	[^{3}H]TdR (cpm/10^6 cells)
I	Saline	—	2·2 (2·3)	4·8 (4·9)	1·8 (1·8)	1·4 (1·4)	6847
	Deoxycytidine	10^{-3}	2·0 (2·1)	5·0 (5·1)	16·0 (16·3)	1·4 (1·4)	1808
	Deoxycytidine	10^{-4}	2·1 (2·2)	5·0 (5·1)	5·2 (5·3)	1·3 (1·3)	3140
	Deoxycytidine	10^{-5}	2·1 (2·2)	4·9 (5·0)	3·0 (3·1)	1·4 (1·4)	4290
II	Saline		5·0 (5·8)	17·0 (17·8)	3·2 (3·4)	1·9 (2·0)	
	Saline		5·0 (5·8)	17·0 (17·8)	3·0 (3·1)	1·9 (2·0)	
	Deoxycytidine	10^{-3}	5·0 (5·8)	17·0 (17·8)	6·6 (6·9)	1·9 (2·0)	
	Deoxycytidine	10^{-4}	5·6 (6·5)	17·0 (17·9)	5·9 (6·2)	1·8 (1·9)	
	Deoxycytidine	10^{-5}	5·0 (5·8)	16·0 (16·8)	3·3 (3·5)	2·1 (2·2)	
	Deoxycytidine	10^{-6}	- --	16·0 -	3·2 —	2·1	
	Deoxycytidine	10^{-7}	5·0 (5·8)	16·0 (16·8)	3·3 (3·5)	2·1 (2·2)	
III	Saline	--	3·5 (3·7)	6·0 (6·2)	3·2 (3·3)	1·2 (1·2)	6266
	Deoxycytidine	10^{-3}	2·7 (2·9)	6·2 (6·4)	7·6 (7·8)	1·0 (1·0)	534

Results are the mean of triplicate experiments.
Numbers in parentheses are corrected for isotope dilution [12].

Table 3. The effect of deoxycytidine (10^{-3} M–10^{-7} M) incubated for 1 hr on the dNTP levels and the incorporation into DNA of [^{3}H]thymidine by PHA-stimulated lymphocytes (72-hr cultures)

Experiment	Drug	Concn (M)	dATP (pmoles/10^6 cells)	dTTP (pmoles/10^6 cells)	dCTP (pmoles/10^6 cells)	dGTP (pmoles/10^6 cells)	[^{3}H]TdR (cpm/10^6 cells)
I	Saline	-	3·5 (3·7)	6·0 (6·2)	3·2 (3·3)	1·2 (1·2)	37662
	Deoxyadenosine	10^{-3}	20 (7·20)	1·3 (1·6)	0 -	0 —	1734
	Deoxyadenosine	10^{-4}	15·8 (16·7)	5·5 (6·4)	1·4 (1·6)	0·7 (0·8)	23609
	Deoxyadenosine	10^{-5}	3·2 (3·3)	5·5 (5·7)	2·7 (2·8)	0·9 (1·0)	40697
II	Saline	—	2·5 (2·6)	4·9 (5·0)	1·7 (1·7)	1·0 (1·0)	8715
	Deoxyadenosine	10^{-3}	45·0 (45·9)	2·2 (3·2)	0·2 (0·3)	0·3 (0·4)	467
	Deoxyadenosine	10^{-4}	3·5 (3·7)	5·5 (5·7)	1·3 (1·3)	1·2 (1·2)	5562
	Deoxyadenosine	10^{-5}	2·4 (2·5)	4·9 (5·0)	1·4 (1·4)	1·2 (1·2)	9365
III	Saline	-	2·3 (2·4)	4·2 (4·3)	1·8 (1·8)	1·2 (1·2)	24118
	Deoxyadenosine	10^{-4}	3·0 (3·1)	4·5 (4·6)	1·2 (1·2)	1·2 (1·2)	1426
	Deoxyadenosine	10^{-5}	2·0 (2·1)	4·8 (4·9)	1·6 (1·6)	1·1 (1·1)	15304

Results are the mean of triplicate experiments.
Numbers in parentheses are corrected for isotope dilution [12].

dNTP levels, even though incorporation of [^{3}H]TdR into DNA was inhibited at all concentrations of deoxycytidine tested (Table 2).

Table 3 shows that deoxyadenosine at concentrations of 10^{-3} M and 10^{-4} M expanded the dATP level and reduced [^{3}H]TdR incorporation into DNA. At 10^{-3} M, there was also a reduction in dTTP, dCTP and dGTP levels, but lower concentrations of deoxyadenosine had no detectable effect on these other dNTP.

Table 4 shows that deoxyguanosine, 10^{-4} M, caused expansion of the dGTP and reduction of the dCTP levels and reduced [^{3}H]TdR incorporation into DNA but had no marked effects on the other dNTP. Lower concentrations of deoxyguanosine had no effect and higher concentrations could not be tested because of insolubility of deoxyguanosine.

DISCUSSION

These results indicate that the external nucleoside concentrations profoundly influence the deoxyribonucleoside triphosphate levels and rate of [^{3}H]thymidine incorporation into DNA in human lymphocytes. Thymidine toxicity in Chinese hamster ovary cells has been associated with depletion of cellular dCTP levels [14] and rescue of the cells with deoxycytidine and these observations have been correlated with the properties of purified ribonucleotide reductase from *E. coli* [7]. A rise of dTTP caused by thymidine has been considered to inhibit ribonucleotide reductase allosterically and produce a failure of reduction of CDP to dCDP. In human PHA-stimulated lymphocytes, thymidine caused expansion of dTTP levels but marked reduction of both dATP and dCTP levels, with a greater effect on dATP. The fall in dATP was not prevented by deoxycytidine. It seems possible that the controls of deoxyribonucleotide synthesis differ in human lymphocytes from those in Chinese hamster ovary cells. Failure of reduction of ADP to dADP rather than failure of reduction of CDP to dCDP may be an important effect of high dTTP concentrations in human lymphocytes but it is not certain that the reduced dATP level caused by thymidine is due to inhibition of ribonucleotide reductase activity. The observation that 10^{-6} M thymidine raised the dTTP concentration in human lymphocytes indicates the extreme sensitivity of these cells to external thymidine. Thymidine levels in mouse plasma have been reported to be 10^{-6} M [15], and in man they are slightly lower [16]. It is, therefore, possible that the plasma levels of thymidine may influence intracellular dNTP levels and proliferation in human cells.

The extreme sensitivity of DNA synthesis in human cells to changes in the dNTP is illustrated by the results with nucleosides other than thymidine, since raising the level of dCTP, dATP or dGTP with the corresponding nucleoside consistently reduced DNA synthesis measured by [^{3}H]TdR incorporation into DNA. The dNTP of human PHA-stimulated lymphocytes showed less sensitivity, however, to the external

Table 4. The effect of deoxyguanosine (10^{-4}–10^{-7} M) incubated for 1 hr on the dNTP levels and the incorporation into DNA of [^{3}H]thymidine by PHA-stimulated lymphocytes

Experiment	Drug	Concn (M)	dATP (pmoles/10^6 cells)	dTTP (pmoles/10^6 cells)	dCTP (pmoles/10^6 cells)	dGTP (pmoles/10^6 cells)	[^{3}H]TdR (cpm/10^6 cells)
I	Saline	-	3·4 (3·6)	5·2 (5·4)	3·2 (3·3)	1·9 (2·0)	37835
	Deoxyguanosine	10^{-4}	3·8 (4·0)	5·0 (5·2)	2·6 (2·7)	11·2 (11·6)	33656
	Deoxyguanosine	10^{-5}	3·4 (3·6)	5·0 (5·2)	2·6 (2·7)	1·8 (1·9)	37465
	Deoxyguanosine	10^{-6}	3·7 (3·9)	5·0 (5·2)	2·6 (2·7)	1·9 (2·0)	39422
	Deoxyguanosine	10^{-7}	4·0 (4·2)	5·2 (5·4)	3·2 (3·3)	1·5 (1·6)	44373
II	Saline	—	3·5 (3·8)	8·6 (8·9)	3·0 (3·1)	1·3 (1·3)	14037
	Deoxyguanosine	10^{-4}	3·9 (4·2)	8·4 (9·1)	2·6 (2·7)	4·9 (5·1)	11525
	Deoxyguanosine	10^{-5}	3·6 (3·9)	8·4 (8·7)	2·8 (2·9)	2·0 (2·1)	14091

Results are the mean of triplicate experiments.
Numbers in parentheses are corrected for isotope dilution [12].

concentration of deoxyadenosine and deoxyguanosine than to those of deoxycytidine and thymidine. This may be related to the high concentration of purines in the cell culture medium but it is known that thymidine kinase activity is high in PHA-stimulated lymphocytes [14], and the relative activities of pyrimidine and purine salvage pathways in these cells may, in part, account for these differences.

At high concentrations, deoxycytidine (10^{-3}–10^{-5} M) caused a rise in dCTP level without any change in the other three dNTP's. These results suggest that dCTP has little importance as a regulator of the synthesis of the other dNTP in human cells. Nevertheless, the rise in dCTP level was accompanied by reduced uptake of tritiated thymidine into DNA suggesting that the imbalance of dNTP reduced cell DNA synthesis. An alternative possibility is that the rise in dCTP had a marked inhibitory effect on thymidine kinase, but this is less likely since the dTTP level was not altered. On the other hand, expanded levels of dATP caused by 10^{-3} M deoxyadenosine decreased dTTP, dCTP and dGTP levels, suggesting that dATP may influence the synthesis of other dNTP's; this is consistent with the observation in bacterial and other mammalian systems that dATP is a potent inhibitor of ribonucleotide reductase. Recovery from inhibition of thymidine incorporation into DNA caused by deoxyadenosine has been reported to be accelerated by deoxyguanosine in bovine liver cells [17]. Although our results indicate that deoxyadenosine causes reduction in the levels of dCTP, dGTP and TTP, it is clear that the inhibition of [^{3}H]thymidine incorporation correlates with the dGTP level most closely. The expansion of dGTP levels caused by 10^{-4} M deoxyguanosine was accompanied by reduced thymidine uptake into DNA. This is consistent with the reports of chromosomal breakage caused by deoxyguanosine but whether this is due to imbalance of dNTP concentrations inhibiting DNA synthesis or another action of a raised dGTP level is uncertain.

These observations that external nucleoside concentration, particularly of the pyrimidines thymidine and deoxycytidine may affect the balance of supply of precursors for DNA synthesis and have a marked effect on the rate of DNA synthesis in phytohaemagglutinin stimulated lymphocytes *in vitro* suggest that the plasma nucleoside concentrations *in vivo* may influence lymphocyte stimulation *in vivo*. It is probable that nucleosides affect DNA synthesis in other cells also. If differential effects of external nucleosides on tumour and normal cell DNA synthesis can be shown, then it may be possible to improve the therapeutic index of antimetabolite drugs given singly or in combination by adjusting the plasma concentration of particular nucleosides by their intravenous administration.

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