Guanine for DNA synthesis

A compulsory route through ribonucleotide reductase

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Two alternative pathways for the synthesis of dGTP and its incorporation into DNA were studied: guanine (Gua) $\rightarrow$ GMP $\rightarrow$ GDP $\rightarrow$ dGDP $\rightarrow$ dGTP $\rightarrow$ DNA and dG $\rightarrow$ dGMP $\rightarrow$ dGDP $\rightarrow$ dGTP $\rightarrow$ DNA. To determine the contribution of each pathway to DNA synthesis independently of each other, [^{14}C]Gua and [^3H]dG tracer experiments were performed in a double-mutant S-49 mouse T-lymphoma cell line, dGuo-L, with purine nucleoside phosphorylase (EC 2.4.2.1)-deficiency and dGTP-feedback-resistant ribonucleotide reductase (RR, EC 1.17.4.1). In this cell line, dGTP pools can be selectively elevated by exogenous dG without affect RR and DNA synthesis. Although [^3H]dG, but not [^{14}C]Gua (up to 200 μM), readily expanded the cellular dGTP pool in a dose-dependent fashion in asynchronous cells, only a small fraction of the Gua flux into DNA was derived from [^3H]dG, with the major fraction coming from [^{14}C]Gua. H.p.l.c. analysis of G_1 - and partially enriched S-phase cells revealed that [^3H]dGTP only accumulates in G_1 - but not in S-phase cells because of a rapid turnover of the dGTP pool during DNA synthesis. These results fail to provide evidence for cellular dGTP compartmentation and suggest that the pathway dG $\rightarrow$ dGMP $\rightarrow$ dGDP $\rightarrow$ dGTP alone has insufficient capacity to maintain DNA synthesis.

INTRODUCTION

Guanine (Gua) depletion by inhibition of IMP dehydrogenase in the 'de novo' pathway of guanine nucleotide synthesis led to a drastic DNA synthesis inhibition before the cellular dGTP pool was exhausted (Cohen *et al.*, 1981). This effect on DNA synthesis appeared to be associated with an extremely small functional precursor dGTP pool that was available for DNA synthesis (Duan & Sadee, 1987; Nguyen & Sadee, 1986). Furthermore, selective repletion of dGTP pool with dG in hypoxanthine:guanine phosphoribosyltransferase (HGPRT)-deficient S-49 cells failed to restore DNA synthesis and only a supply of Gua ribonucleotides reversed the inhibition (Cohen *et al.*, 1981; Nguyen *et al.*, 1983). Hence, we postulated that the dGTP precursor pool for DNA synthesis is sequestered in a small cellular compartment (Nguyen & Sadee, 1986). Cellular compartmentation of nucleotide pools has been postulated (Mathews & Slaugh, 1986), and Reddy & Pardee (1980) have proposed the presence of a multienzyme complex for channelling deoxynucleoside triphosphates into DNA.

In mammalian cells, dGTP can be derived from either the 'de novo' synthesis pathway or the salvage pathways as depicted in Scheme 1. Gua, and through the action of PNP, G and dG, can provide cells with dGTP via the ribonucleotide intermediates and though the enzyme ribonucleotide reductase (RR), as in the case of 'de novo' synthesis, while only dG can be directly phosphorylated to Gua deoxyribonucleotides (dG $\rightarrow$ dGMP $\rightarrow$ dGDP $\rightarrow$ dGTP) (Nguyen & Sadee, 1986; Weber, 1983). In an attempt to quantify the contributions of Gua-DNA by the two pathways, which could feed into separate cellular dGTP pools, we here utilize a double-mutant S-49 mouse

T-lymphoma cell line, dGuo-L, with purine nucleoside phosphorylase (PNP) deficiency and partial RR resistance to dGTP feedback inhibition (Ullman *et al.*, 1979). The use of dGuo-L circumvents problems previously encountered in using PNP-deficient single-mutant cells (Nguyen & Sadee, 1986), which are highly sensitive to dG and to dGTP feedback inhibition of RR and hence inhibition of DNA synthesis. With dGuo-L cells, we were able to increase dGTP levels significantly by adding exogenous dG, without critically affecting RR activity, in order to compare the effectiveness of the direct phosphorylation pathway with the RR pathway in providing dGTP for DNA synthesis.

MATERIALS AND METHODS

Materials

dG, Gua and Bt_2cAMP ($N^6,2'$ -*O*-dibutyl cyclic AMP) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Mycophenolic acid was obtained from Eli Lilly and Co., Indianapolis, IN, U.S.A. [$8\text{-}^{14}\text{C}$]Gua (57 mCi/mmol) and [$8\text{-}^3\text{H}$]dG (5 Ci/mmol) were purchased from ICN Pharmaceuticals, Irvine, CA, U.S.A. [^3H]2-dT (80 Ci/mmol) and [^{14}C]U (23 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. All other reagents were of the highest grade available.

Cell culture

S-49 mouse T-lymphoma wild-type, and two cloned mutants, the PNP-deficient mutant (NSU-1) and dGuo-L cells, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated horse serum in a 10% (v/v) CO_2 incubator at 37 $^\circ\text{C}$

Abbreviations used: Gua, guanine; PNP, purine nucleoside phosphorylase; HGPRT, hypoxanthine:guanine phosphoribosyltransferase; RR, ribonucleotide reductase; Bt_2cAMP , $N^6,2'$ -*O*-dibutyl cyclic AMP.

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Scheme 1. 'De novo' and salvage pathways of guanine nucleotide synthesis

Enzymes shown the 'de novo' pathway are: IMP-DH, IMP dehydrogenase; and RR, ribonucleotide reductase; and those in the salvage pathways are: dCK, deoxycytidine kinase; HGPRT, hypoxanthine:guanine phosphoribosyl-transferase; and PNP, purine nucleoside phosphorylase.

(Ullman *et al.*, 1979). Lack of significant incorporation of [^3H]dG into GTP of the two PNP-deficient mutants was taken as evidence that the PNP deficiency was unaltered in the cells used here.

For G_1 cell-cycle-phase enrichment, exponentially growing S-49 cells were incubated with Bt_2cAMP (500 μM) for 24 h. S-phase-enriched cells were obtained 24 h after removal of Bt_2cAMP from medium (Albert & Gudus, 1985).

Cell cycle analysis

Cell suspensions of 1×10^6 cells/ml fixed with 70% (v/v) ethanol were centrifuged and stained with chromomycin A_3 (1.7×10^{-5} M) solution containing MgCl_2 (0.015 M) for 1 h. Cells were then filtered through nylon mesh (50 μm diameter) and analysed on an FACS III flow cytometer at 457 nm. The fluorescence emission was recorded using a 520 nm long-pass filter (Crissman & Tobey, 1974).

Tracer incorporation into DNA

Exponentially growing S-49 cells (2×10^5 /ml) were incubated with 1 μCi of [^3H]dG (various concentrations) at 37 $^\circ\text{C}$ for 4 h. Cells were then harvested, washed with ice-cold phosphate-buffered saline and precipitated with 0.4 M ice-cold perchloric acid. The supernatants were saved for h.p.l.c. analysis of nucleotides and the pellets were washed one more time with 0.4 M cold perchloric acid. KOH (0.3 M) digestion of RNA, separation of DNA from RNA and evaluation of radioactivity were performed as previously described (Duan & Sadee, 1987).

Analysis of nucleotide pools

Perchloric acid supernatants were extracted with

Freon/n-octylamine (39:11, v/v) and analysed on h.p.l.c. for nucleotides with a Partisil 10-SAX column (25 cm $\times$ 4.6 mm) and a linear elution gradient of 0.02 M- $\text{NH}_4\text{H}_2\text{PO}_4$ in 0.01 M-KCl, pH 3.6 (A), and 1.0 M- $\text{NH}_4\text{H}_2\text{PO}_4$ in 0.5 M-KCl, pH 3.6 (B), (A $\rightarrow$ 100% B in 20 min) at a flow rate of 2 ml/min (Pogolotti & Santi, 1982). Eluted materials were recorded with a u.v. detector at 254 nm. External nucleotide standards (2 μmol each) were used to access nucleotide pool sizes. Eluant fractions (0.3 ml) were collected for analysis of radioactivity profile in nucleotide pools.

RESULTS

Incorporation of Gua or dG alone into DNA

When exponentially growing dGuo-L cells (2×10^5 cells/ml) were incubated with (10 μM)-mycophenolic acid (which blocks 'de novo' guanine nucleotide synthesis and DNA synthesis) and various concentrations of [^{14}C]-Gua for 4 h, the Gua incorporation (corrected for ^{14}C specific radioactivity) into DNA increased linearly with Gua concentration and plateaued at 200 μM -Gua (Fig. 1). Further experiments showed that mycophenolic acid was not necessary, since Gua alone at 200 μM completely inhibited 'de novo' GMP synthesis. The incorporation of [^{14}C]Gua into DNA under identical conditions was previously shown to be linear with time over 4 h (Nguyen & Sadee, 1986), which permits the determination of absolute DNA synthesis rates, assuming negligible dilution with endogenous Gua under these conditions. The plateau incorporation of Gua was 14 nmol/30 min per 10^8 cells (Fig. 1), which was slightly higher than the reported DNA synthesis rate of ~ 9 nmol of dG/30 min per 10^8 cells in S-49 wild-type cells (Maybaum *et al.*, 1981).

Similarly, in the presence of mycophenolic acid (10 μM) and the absence of exogenous Gua, the incorporation of exogenous [^3H]dG into DNA of dGuo-L cells was proportional to dG concentration up to 300 μM (~ 12 nmol/30 min per 10^8 cells at 300 μM -dG, Fig. 1b). In the same

Fig. 1. Incorporation of Gua or dG into DNA of dGuo-L cells

dGuo-L cells (2×10^5 /ml) were preincubated with 10 μM -mycophenolic acid for 1 h and [^{14}C]Gua (a) or [^3H]dG (b) was then added to the cultures for 4 h before harvesting. Error range for each point is $\leq 5\%$ ($n = 4$).

Table 1. Accumulation from exogenous dG

dGuo-L cells (2×10^5 /ml) were incubated with dG for 4 h and harvested for h.p.l.c. analysis of nucleotide pools

[dG] (μ M)	dGTP (nmol/ 10^8 cells)
0	1.8
25.0	5.3
50.0	8.9
100.0	13.0
300.0	25.0

experiments, h.p.l.c. analysis showed that dGTP pool size in dGuo-L cells increased from 1.8 nmol/ 10^8 cells to 25 nmol/ 10^8 cells at 300 μ M-dG (Table 1), while Gua failed to increase significantly the dGTP pool at concentrations as high as 200 μ M. It should be noted, however, that dG at ≥ 100 μ M underwent sufficient conversion to Gua and GTP so that at least part of the Gua-DNA flux was not derived from the direct phosphorylation pathway (see [3 H]dGuo data below).

Competition between Gua and dG for incorporation into DNA

To address the question whether dGTP from the direct phosphorylation pathway (from dG) can compete with dGTP derived from pathways involving RR ('*de novo*' or from Gua) for incorporation into DNA, the simultaneous incorporation of [14 C]Gua and [3 H]dG into DNA of dGuo-L cells was investigated. dGuo-L were incubated

Fig. 2. Competition for incorporation into DNA between dG and Gua

dGuo-L cells (2×10^5 /ml) were incubated with 200 μ M- [14 C]Gua and various concentrations of the amounts of [3 H]dG for 4 h. The incorporation of [14 C]Gua ($\blacktriangle$) and [3 H]dG ($\bullet$) and the sums of the amounts of [14 C]Gua and [3 H]dG incorporated are shown ($\blacksquare$). Error range for each point is $\leq 5\%$ ($n = 4$).

Table 2. Incorporation of [3 H]dG and [14 C]Gua into nucleotide pools and DNA

dGuo-L cells were incubated with 200 μ M- [14 C]Gua and 50 μ M- [3 H]dG for 4 h. After harvesting, nucleotide pools and radiolabelled profile were analysed by h.p.l.c. and dual-label-scintillation counting. This experiment was repeated four times with similar results.

	3 H	14 C
GTP (d.p.m.)/ 10^6 cells)	180 ± 20	2750 ± 320
dGTP (d.p.m./ 10^6 cells)	5190 ± 440	340 ± 50
DNA (% total d.p.m.)	12 ± 2	88 ± 2

with 200 μ M- [14 C]Gua and various concentrations of [3 H]dG for 4 h. Calculations on the basis of specific radioactivities of tracers showed that the incorporation of [3 H]dG into DNA increased, while that of [14 C]Gua decreased with dG concentration, with the sums of incorporation of both tracers unchanged (~ 12 nmol/30 min per 10^8 cells) at any given dG concentration (Fig. 2). However, h.p.l.c. analysis of nucleotide pools showed that 3 H radioactivity in GTP significantly increased at dG concentrations ≥ 100 μ M, indicating extensive conversion of dG to Gua by either residual PNP in dGuo-L cells, PNP in the medium, or both. Therefore, under conditions where 3 H was mainly derived from the direct phosphorylation pathway of [3 H]dG, i.e. at dG concentrations ≤ 50 μ M, dGTP from dG only contributed $\leq 12\%$ of the total Gua-DNA even though dGTP levels were increased 5-fold by 50 μ M- [3 H]dG. While dG inhibited cellular growth of dGuo-L cells with an IC_{50} of ~ 150 μ M (Martin & Gelfand, 1981; our results), 50 μ M-Gua had only minimal effects on dGuo-L cells, and no measurable effects on DNA synthesis.

Labelling of GTP and dGTP of dGuo-L cells by [14 C]Gua and [3 H]dG

As shown in Table 2, after incubation of dGuo-L cells with 200 μ M- [14 C]Gua and 50 μ M- [3 H]dGuo for 4 h,

Table 3. dGTP labelling profile of synchronized dGuo-L cells

Bt₂AMP-synchronized dGuo-L cells were incubated with 200 μ M- [14 C]Gua (1 μ Ci) and 50 μ M- [3 H]dG (1 μ Ci) for only 1 h (to avoid large changes in cell distribution among different cell-cycle phases) and harvested for nucleotide analysis. This experiment was repeated three times with similar results.

	G ₁ -phase (95-3-2)*	S-phase (42-55-3)*
14 C (d.p.m./ 10^8 cells)	< 1000	< 1000
3 H (d.p.m./ 10^8 cells)	243800 ± 4500	59425 ± 620
dGTP pool size (nmol/ 10^8 cells)	8.4 ± 0.3	3.9 ± 0.1

* % Total cells in G₁-, S- and G₂/M-phase respectively.

[¹⁴C]Gua specifically accumulated in GTP with negligible labelling of dGTP, while [³H]dG specially labelled dGTP, indicating little cross-over of ³H into the RR pathway. Meanwhile, the control wild-type S-49 cells actively incorporated ³H from [³H]dG into GTP via PNP under identical conditions. In contrast, ³H (from direct phosphorylation) of [³H]dG contributed only 12 % of the total Gua flux into DNA of dGuo-L cells, while ¹⁴C (from [¹⁴C]Gua via RR) accounted for 88 % (Table 2). We have also studied GTP and dGTP labelling by 200 μ M-[¹⁴C]Gua and 50 μ M-[³H]dG in dGuo-L cells partially synchronized with 0.5 mM-Bt₂cAMP. In S-phase (55 % S)-enriched dGuo-L cells, the level of ³H label in dGTP was depressed to one-quarter that in G₁-phase (95 % G₁) cells (Table 3). The pool size of dGTP was 8.4 ± 0.3 nmol/10⁸ cells in G₁-phase cells (by h.p.l.c. measurement) and 3.9 ± 0.1 nmol/10⁸ cells in S-phase-enriched dGuo-L. Thus the dGTP pool was considerably lower in S-phase than in G₁-phase cells when exogenous dGuo was present.

DISCUSSION

We have investigated the contribution of dGTP to DNA synthesis from two dGTP-synthesizing pathways in the S-49 cell line dGuo-L, which is PNP-deficient and carries an RR partially resistant to dGTP-feedback inhibition (Ullman *et al.*, 1979). Because of the partial resistance to dGTP-feedback inhibition of RR, dGuo-L cells were able to accumulate high levels of dGTP from exogenous dG through the direct phosphorylation pathway (~5-fold normal dGTP at 50 μ M-dG) without affecting DNA synthesis. [¹⁴C]Gua (200 μ M), used to supply dGTP via the RR pathway, failed to elevate dGTP pools. Despite the ability of asynchronous dGuo-L cells to accumulate high levels of dGTP from exogenous dG, dGTP from the direct phosphorylation route (dG → dGMP → dGDP → dGTP) contributed only ~12 % of total Gua in DNA at 50 μ M-dG, while the rest of Gua-DNA came from [¹⁴C]Gua via RR (Gua → GMP → GDP → dGDP → dGTP). Thus dGTP for DNA synthesis appeared to be derived mainly from pathways involving RR when Gua nucleotide precursors from both pathways were available. Two hypotheses can resolve this paradox: first, there is extensive cellular compartmentation which produces a small functional dGTP precursor pool with direct access to DNA synthesis; and secondly, dGTP accumulation by exogenous dGuo only occurs in G₁, but not S-phase, cells. Consistent with the second hypothesis, exogenous dG caused a 4-fold higher accumulation of [³H]dGTP in G₁- than in S-phase-enriched dGuo-L cells. Considering the contamination of G₁-phase cells in the S-phase-enriched cell population, the accumulation of dGTP from dG in S-phase cells was markedly lower than that in G₁-phase cells. It has been previously reported that RR is only active during the S-phase of the cell cycle (Albert & Gudus, 1985) during ongoing DNA synthesis, while deoxycytidine kinase (the major kinase species that phosphorylates dG) is active throughout the cell cycle (Eriksson *et al.*, 1984). Thus our results are consistent with the hypothesis that G₁-phase dGuo-L cells extensively accumulate dGTP exclusively from dG, because

RR is inactive and dGTP turnover is small. Once into S-phase, the dGTP turnover increases dramatically because of dGTP utilization for DNA. Furthermore, RR is activated and deoxycytidine kinase, with a very high K_m for dG (180 μ M; Krenitsky *et al.*, 1976), can no longer compete with RR in providing dGTP for DNA synthesis. The lack of ¹⁴C accumulation in dGTP, while ¹⁴C from [¹⁴C]Gua is preferentially incorporated into DNA, reflects the high dGTP turnover in S-phase cells. These results provide no evidence for cellular dGTP compartmentation or a major role for a multienzyme complex that channels dGTP into DNA (Reddy & Pardee, 1980). These results also account for the observations that dG is incapable of supporting DNA synthesis after blocking IMP dehydrogenase *de novo* by mycophenolic acid, unless very high dG levels are employed (together with impairment of dGTP-RR-feedback inhibition as in dGuo-L). However, high levels of dG (≥ 100 μ M) are toxic to dGuo-L cells by yet unknown alternative mechanisms. Furthermore high dG concentrations partially overcome the PNP deficiency in dGuo-L cells and provide a source of Gua ribonucleotides.

The results reported here are similar to those obtained by Leeds & Mathews (1987) with dC, and from our laboratory with dA (Duan & Sadee, 1987), that also document a considerably higher turnover of dCTP and dATP during the S-phase of the cell cycle, which accounts for anomalies of tracer incorporation into deoxynucleoside triphosphate and DNA in asynchronous cell culture.

This study was supported by Grant CA 27866 from the National Cancer Institute.

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