Mechanisms of 2'-deoxyguanosine toxicity in mouse T-lymphoma cells with purine nucleoside phosphorylase deficiency and resistance to inhibition of ribonucleotide reductase by dGTP

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Purine nucleoside phosphorylase (PNP; EC 2.4.2.1) deficiency is thought to cause T-lymphocyte depletion by accumulation of dG and dGTP, resulting in feedback inhibition of ribonucleotide reductase (RR; EC 1.17.4.1) and hence DNA synthesis. To test for additional toxic mechanisms of dG, we selected a double mutant of the mouse T-lymphoma S-49 cell line, dGuo-L, which is deficient in PNP and partially resistant to dGTP feedback inhibition of RR. The effects of dG on dGuo-L cells (concn. causing 50 % inhibition, $IC_{50} = 150 \mu M$) were compared with those on the wild-type cells ($IC_{50} = 30 \mu M$) and the NSU-1 mutant with PNP deficiency only ($IC_{50} = 15 \mu M$). Fluorescence flow cytometry showed that equitoxic dG concentrations arrested wild-type and NSU-1 cells at the G_1 -S interface while allowing continued DNA synthesis in the S-phase, whereas the double mutant dGuo-L cells progressed through the cell cycle normally. dGuo-L cells accumulated high levels of dGTP in G_1 -phase, but not in S-phase cells, because of the utilization of dGTP for DNA synthesis and limited capacity to synthesize dGTP from dG. These results support the hypothesis that dG/dGTP toxicity occurs in the G_1 -phase or at the G_1 -S interface. Failure of dG to arrest the double mutant dGuo-L cells at the G_1 -S interface allows these cells to escape into S-phase, with an accompanying drop in dGTP levels. Thus the partial resistance of dGuo-L cells to dG toxicity may result from their shorter residence time in G_1 , allowing them to sustain higher dGTP levels. Hence RR inhibition by dGuo may not be the primary toxic mechanism in S-49 cells; rather, it may serve as an accessory event in dG toxicity by keeping the cells in the sensitive phase of the cell cycle. Among the possible targets of dG toxicity is RNA synthesis, which was inhibited at an early stage in dGuo-L cells.

INTRODUCTION

An inborn deficiency of purine nucleoside phosphorylase (PNP; EC 2.4.2.1) results in severe immunodeficiency that is characterized by marked T-lymphocyte depletion and normal B-lymphocyte functions [1,2], in contrast with a combined severe immunodeficiency caused by adenosine deaminase deficiency, in which both B- and T-lymphocyte functions are affected [3,4]. Whereas multiple mechanisms have been reported for ADA deficiency ([5] and refs. therein), the predominant cause of T-lymphocyte toxicity in PNP deficiency is thought to be the accumulation of dG and its metabolite, dGTP [6,7], which causes feedback inhibition of ribonucleotide reductase (RR; EC 1.17.1.1) activity towards CDP [5,8] and enhances ADP reduction [9]. The selectivity of dG cytotoxicity against T-lymphocytes may rest in the ability of these cells to trap dGTP inside the cell by high kinase and low nucleotidase activities [10,11]. dGuo and its analogues are therefore considered to be potential chemotherapeutic agents for leukaemias [12]. However, the hypothesis of RR inhibition by dGTP failed to explain the early toxicity caused by rather high dG levels before initiation of DNA synthesis in concanavalin-A-stimulated murine spleen T-lymphocytes [13], the selective sparing of T helper cell activity required for normal B-lymphocyte function [14,15], or the inability of deoxycytidine to reverse dG effects on phytohaemagglutinin-stimulated human peripheral blood lymphocytes [11,16]. Finally, several studies on dG toxicity were performed in cells with normal PNP activity, allowing for dG conversion to guanine and subsequent guanine toxicity (e.g. [8,17]). Therefore

the purpose of this study is to address additional mechanisms of dG that may have implications in its immunological effects and cytotoxicity.

To investigate cytotoxic effects of dG that are independent of feedback inhibition of RR, we selected a mouse T-lymphoma cell line, dGuo-L [18]. This double-mutant of the parent S-49 mouse cells is devoid of PNP and heterozygous for an RR allele resistant to dGTP feedback regulation [18]. The results were compared with those obtained in the parent S-49 wild-type cells and in PNP-deficient mutant cells from which the mechanism involving RR inhibition was derived [5]. Our results suggest that RR inhibition may not be the primary mechanism of dG toxicity against S-49 cells, but rather serves to maintain cells in the sensitive phase, G_1 or G_1 -S interphase, of the cell cycle.

MATERIALS AND METHODS

Materials

5-Bromodeoxyuridine (BrdU), chromomycin A₃, dG, *N*⁶,2'-*O*-dibutyl cyclic AMP (Bt₂cAMP), ethidium bromide, Freon (1,1,2-trichloro-1,2,2-trifluoroethane), iodoacetic acid, propidium iodide, proteinase K, tri-*n*-octylamine, Tris buffer and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [5-³H]Cytidine ([³H]C, 23 Ci/mmol), [8-³H]dG (7.4 Ci/mmol), [8-¹⁴C]guanine ([¹⁴C]Gua, 57 mCi/mmol), [2,3,4,5-³H]leucine (19 Ci/mmol), [*methyl*-³H]dT (80 Ci/mmol), and [5-³H]uridine ([³H]U, 23 mCi/mmol) were purchased from ICN Pharmaceutical Inc., Irvine, CA, U.S.A. Mouse anti-BrdUrd antibody was kindly supplied by Dr. Joe Gray, National

Abbreviations used: BrdU, 5'-bromodeoxyuridine; Bt₂cAMP, *N*⁶,2'-*O*-dibutyl cyclic AMP; [¹⁴C]Gua, [8-¹⁴C]guanine; IC_{50} , concentration causing 50 % inhibition; PBS, phosphate-buffered saline; PNP, purine nucleoside phosphorylase; RR, ribonucleotide reductase.

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Lawrence Livermore Laboratory, Livermore, CA, U.S.A. Vanadyl-ribonucleotide complex was purchased from BRL Life Technology, Gaithersburg, MD, U.S.A. SDS, electrophoresis-grade agarose and urea were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other reagents were of analytical grade.

Cell culture

S-49 wild-type, NSU-1 (PNP-deficient) mutant and dGuo-L mutant cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated horse serum at 37 °C in 10% CO₂. The growth characteristics of these cells have been described elsewhere [18]. For growth experiments, dG was added to exponentially growing cells (5×10^5 /ml) for 24 h. Cell counts were then determined with a Coulter counter model Z₁. For G₁-phase enrichment, S-49 cells were incubated with 500 μ M-Bt₂cAMP for 24 h and washed with phosphate-buffered saline (PBS; 10 mM-sodium phosphate, 120 mM-NaCl and 2.7 mM-KCl, pH 7.4) immediately before experiments. S-phase-enriched cell populations were obtained 24 h after washing of Bt₂cAMP.

Deoxyribonucleotide pool determination

S-49 cells incubated with dG for 4 h were harvested and washed with ice-cold PBS. Ice-cold HClO₄ was added to the cells, and the supernatant was extracted with a mixture of Freon and tri-n-octylamine (78:22, v/v). The aqueous extract was analysed by h.p.l.c. for nucleotide contents using a linear gradient solvent system of 0.02 M-NH₄H₂PO₄ in 0.01 M-KCl, pH 3.6 (buffer A) and 1.0 M-NH₄H₂PO₄ in 0.5 M-KCl, pH 3.6 (buffer B), starting with 100% B in 20 min at a flow rate of 2.0 ml/min. External standards of 2–5 μ mol of each nucleotide were used for quantification. Fractions of 0.6 ml were collected for the desired peaks and assayed for radioactivity by scintillation counting [19].

DNA, RNA and protein synthesis determination

Cells growing logarithmically ($\sim 5 \times 10^5$ /ml) were incubated with dG and 1 μ Ci/ml concentrations [³H]C, [³H]dT, [³H]U or [¹⁴C]Gua, in combination with [³H]dG, for 4 h at 37 °C. After washing with PBS, pellets were precipitated with ice-cold HClO₄ (0.4 M), washed twice with HClO₄ and digested with 0.3 M-KOH overnight [20]. Counts in the final HClO₄ precipitate and in the supernatant represent incorporation of tracers into DNA and RNA respectively.

For protein synthesis measurement, cells were incubated with dG, and [³H]Leu (1 μ Ci/ml) was added 2 h before harvest. The HClO₄ pellets were washed and then harvested on GF-B filter paper followed by three washes with ice-cold HClO₄.

Cell cycle analysis

Cell suspensions containing approx. 10^6 cells were centrifuged and fixed in 1 ml of 70% ethanol and stored at 4 °C overnight. Cells were then harvested and stained with chromomycin A₃ (17 μ M) solution containing MgCl₂ (0.015 M) for 1 h before analysis on a 5-W argon-laser FACSIII flow cytometer (Becton-Dickinson, Sunnyvale, CA, U.S.A.) at 457 nm [21].

For BrdU/propidium iodide dual staining, a modified procedure according to Dolbeare *et al.* [22] was used. Briefly, cells were pulsed with a subtoxic concentration of BrdU (10 μ M) for 30 min to label the DNA of S-phase cells, and then incubated with dG. Harvested cells ($\sim 5 \times 10^6$) were fixed in ethanol and resuspended in 1.5 ml of cold HCl (0.1 M) containing 0.7% Triton X-100. Cells were pelleted, and DNA was denatured by incubating with 2.4 M-HCl at room temperature for 20 min. After washing with buffer (0.5% Tween 20 in PBS), 500 μ l of mouse anti-BrdU antibody (IU:4, diluted 1:4000 in PBS/0.5% Tween 20/0.5% BSA) was added to the cells and incubated for

30 min at room temperature. A second antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody, was added for 20 min. Washed pellets were then stained with 1 ml of propidium iodide (10 μ g/ml in PBS) for 30 min, syringed through a single-cell nylon mesh (22 μ m) and analysed directly using a dual-laser FACSII flow cytometer. To compare the rates at which BrdU-labelled control and dG-treated cells progress through the cell cycle, the percentage of BrdU-labelled cells in the G₁-, S-, and G₂/M-phases at different sampling times was calculated as described [22]. Further, DNA synthesis time (T_s) was also calculated for both control and dG-treated cells at different times of sampling, as previously described by Begg *et al.* [23].

RNA synthesis in cell homogenates

Exponentially growing dGuo-L cells were harvested by centrifugation, washed twice with cold PBS and resuspended in a buffer containing 150 mM-sucrose, 80 mM-KCl, 35 mM-Hepes, 5 mM-potassium phosphate and 5 mM-MgCl₂, pH 7.4, at 2×10^8 cells/ml. Portions of the cell suspension (0.8–1 ml) were homogenized with a Brunschwig Ultrasonic cell disrupter (micro tip) with 60 pulses (20% active cycle) at power output level 2. Cell homogenate (25 μ l) was added to 75 μ l of reaction mixture in a buffer containing 50 mM-Hepes, 7.5 mM-potassium phosphate and 10 mM-MgCl₂, pH 7.4, giving final concentrations of 1 mM-dithiothreitol, 0.5 mM-ATP, -CTP and -UTP, and 0.1 mM-[³H]GTP (specific radioactivity 222 d.p.m./pmol). Alternatively, 1 mM-dithiothreitol, 0.5 mM-ATP, -CTP and -GTP and 0.1 mM-[³H]UTP were used. The reaction mixture was supplemented with 0–2.5 mM-dGTP or -dATP and incubated at 37 °C for 1–60 min. RNA- or polynucleotide-bound radioactivity was determined as described [24].

Isolation and electrophoresis of cytoplasmic RNA

[³H]Gua-labelled cells were washed with ice-cold PBS and resuspended in iced lysis buffer C (10 mM-Tris, pH 7.0, 10 mM-NaCl, 0.2% Triton X-100, 1% ethanol and 100 mM-vanadyl-ribonucleotide complex). The mixture was vortex-mixed and kept on ice for 10 min. After centrifugation, the supernatant (containing cytoplasmic RNA) was removed, and 0.2 vol. of lysis buffer D (100 mM-Tris/HCl, 50 mM-EDTA and 10% SDS, pH 7.1) was added. The solution was incubated with proteinase K (final concentration 200 μ g/ml) at 37 °C for 30 min followed by three extractions with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). Extracted cytoplasmic RNA was stored in 100% ethanol containing 0.2 M-NaCl at –20 °C overnight before analysis. Electrophoresis of [³H]RNA was performed using vertical 1.5% agarose gels containing 6 M-urea and 0.015 M-iodoacetate in a TPE running buffer (0.04 M-Tris, 0.036 M-NaH₂PO₄, and 0.001 M-EDTA, pH 7.4) at constant voltage (40 V) for 12 h. RNA bands were then revealed by staining with ethidium bromide. Incorporation of ³H into different size classes of RNA was determined by fractionation of the gels and liquid scintillation counting of the gel slices.

RESULTS

Inhibition of S-49 cell growth by dG

Over a 24-h incubation, dG inhibited growth of wild-type S-49, PNP-negative (NSU-1) and the double-mutant (dGuo-L) cells, with IC₅₀ values (concns. causing 50% inhibition) of ~ 30 μ M, 15 μ M and 150 μ M respectively. These results were similar to values reported by Ullman *et al.* [18], who attributed the lower sensitivity of dGuo-L cells to a partial loss of feedback inhibition of RR by dGTP.

Fig. 1. dGTP accumulation in synchronized S-49 cells

To yield G₁-phase cells (●), PNP-deficient mutant (NSU-1, a) and the double-mutant (dGuo-L, b) cells were synchronized using Bt₂cAMP (0.5 mM) (95% and 92% respectively). S-phase-enriched cell populations (■) (58% and 64% respectively) were also obtained. The cells were labelled with [³H]dG (1 μCi/ml) for only 1 h to minimize changes in cell-cycle distribution. Incorporation of ³H into dGTP was measured by h.p.l.c. after harvesting. Each point represents the mean of duplicates ± s.d.

Accumulation of dGTP from dG by dGuo-L cells

In order to test whether the less sensitive dGuo-L cells accumulate dGTP when exposed to dG, dGTP pools were measured by h.p.l.c. After 4-h incubations, dGTP levels in dGuo-L cells increased by 3–5-fold with low dG concentrations (25 μM) and up to 15-fold at 300 μM-dGuo, compared with controls (for detailed kinetic studies, see [25]). The accumulation of dGTP by dGuo-L cells was similar to or exceeded by up to 2-fold the dGTP accumulation in wild-type and PNP-deficient cells. The partial resistance of dGuo-L cells was therefore not associated with failure to phosphorylate dG to dGTP.

Failure of dGTP accumulation in S-phase cells

The kinetics of [³H]dGTP were then studied in partially synchronized S-49 cells. After treatment with 0.5 mM-Bt₂cAMP for 24 h, S-49 wild-type, NSU-1 and dGuo-L cells were synchronized at the G₁-phase (> 95%) of the cell cycle. These cells were then released from G₁-phase by resuspension in fresh medium, and S-phase-enriched cells (50–60% of cells in S-phase, determined by flow cytometry) were obtained 24 h later [26]. When G₁-phase or S-phase-enriched S-49 wild type cells were labelled with [³H]dG tracer, ³H activity was found primarily in GTP, as a result of high PNP activity. In contrast, [³H]dG predominantly labelled the dGTP pool of the PNP-deficient mutants, i.e. NSU-1 and dGuo-L cells, which is expected from lack of metabolic conversion of the tracer to [³H]Gua (see also [25]). Surprisingly, however, [³H]dG labelled the dGTP pool up to 3-fold more efficiently in G₁-phase- than in S-phase-enriched cells (Fig. 1).

These results suggested that G₁-phase cells preferentially accumulate high dGTP levels when exposed to dG, whereas dGTP accumulation is prevented in S-phase because of ongoing DNA synthesis. Therefore the dGTP pool expansion by dG

(50 μM, plus 1 μCi of [³H]dG for 1 h) was tested by h.p.l.c. in synchronized dGuo-L cells. The dGTP pool size in G₁-phase cells increased from ~0.8 nmol/10⁸ cells in the control to ~8.5 nmol/10⁸ cells, and the measured specific radioactivity of dGTP was identical to that of [³H]dG added to the medium. In contrast, S-phase-enriched (55%) dGuo-L cells reached a dGTP pool of only ~3.5 nmol/10⁸ cells, with a specific radioactivity of [³H]dGTP only 45% of that of the added [³H]dG. Similar experiments with NSU-1 cells confirmed the 2–3-fold lower pool sizes of dGTP in S-phase-enriched compared with G₁-phase cells. The tracer dilution in the dGTP pool indicates that unlabelled dGTP was formed from unlabelled GDP precursors via RR, which is active in S-phase but not in G₁-phase cells [26]. If one further considers the presence of approx. 45% labelled G₁-phase cells in the S-phase-enriched cell population, it becomes clear that S-phase cells accumulated negligible amounts of [³H]dG into dGTP and that the overall dGTP pool size remained rather small.

DNA and RNA synthesis by S-49 cells

Because dG can cause perturbations of nucleotide metabolism, we used several radioactive tracers to assess the effects of dG on DNA and RNA synthesis by S-49 cells. After 4 h of incubation, [³H]dT incorporation was slightly decreased by dG in wild-type cells (60 μM, 95% of control) and NSU-1 cells (40 μM, 67% of control). Longer incubation periods up to 12 h further decreased [³H]dT incorporation to around 30% of the control, followed by partial recovery at 24 h (~50% of control) in both cell lines. In contrast, [³H]dT incorporation into the DNA of dGuo-L cells was actually enhanced by dG (up to 2.5-fold at 350 μM), which can be attributed to changes in dTTP turnover; however, these results argue against a drastic inhibition of DNA synthesis in dGuo-L cells. [³H]C was then used to label both RNA and DNA, the latter involving the RR-mediated conversion of CDP to dCDP (Table 1). Again, there was only a rather small reduction of [³H]C incorporation into DNA in the dGuo-L cells. In contrast, [³H]C labelling of DNA was more strongly decreased by dG in S-49 wild-type and NSU-1 mutant cells. Although the fall to 30% of control DNA labelling suggests inhibition of DNA synthesis, one might have expected a stronger inhibition of tracer incorporation if accumulated dGTP strongly inhibits RR. Apparently, residual RR activity and DNA synthesis continued over at least 4 h of dG exposure. The incorporation of both [³H]C and [³H]U into the RNA of dGuo-L cells indicated depression (~50%) of RNA synthesis in all three cell lines (Table 1).

To validate the effects of dG on DNA and RNA synthesis by dGuo-L cells, simultaneous incorporation of [³H]dG and [¹⁴C]Gua into DNA and RNA was measured. dGuo-L cells were incubated for 4 h with 10 μM-mycophenolic acid (to inhibit inosine 5'-monophosphate dehydrogenase), 200 μM-[¹⁴C]Gua (to maintain > 90% normal DNA synthesis) [25], and various concentrations of [³H]dG. Mycophenolic acid served to prevent any formation of guanine nucleotides *de novo*, and high concentrations of the diluted tracer (1 μCi/ml of each) were chosen to minimize further dilution by endogenously preformed guanine nucleotides. Subsequent experiments showed that the presence of mycophenolic acid had little effect on tracer flux and incorporation, since the addition of 200 μM-Gua was sufficient to fully suppress GMP formation *de novo*. Control experiments with [¹⁴C]Gua (200 μM) in 4 h incubations gave DNA synthesis rates similar to the calculated absolute rate of ~9 nmol dG/30 min per 10⁸ cells for S-49 cells [27]. Calculations of the sum of dG and Gua incorporated into DNA and RNA, on the basis of specific radioactivities of the added tracers, showed that the rate of DNA synthesis was not affected in dGuo-L cells by increasing con-

Table 1. Effects of dG on the incorporation of [³H]dT, [³H]U and [³H]C into dGuo-L-cells

Cells were incubated with dG together with [³H]dT and [³H]U for 4 h, or labelled with [³H]C for 1 h after a 4 h incubation with dG before harvesting. DNA and RNA were separated and radioactivity determined. Results are means $\pm$ S.D., $n = 2$.

[dG] (μ M)	dGuo-L cells			[dG] (μ M)	Wild-type cells		[dG] (μ M)	PNP-deficient cells	
	[5- ³ H]U incorporation into RNA (% of control)	[5- ³ H]C incorporation (% of control)			[5- ³ H]C incorporation (% of control)	[5- ³ H]C incorporation (% of control)			
		DNA	RNA					DNA	RNA
0	100 ± 6	100 ± 1	100 ± 2	0	100 ± 2	100 ± 1	0	100 ± 3	100 ± 1
25	93 ± 1	104 ± 5	89 ± 4	10	82 ± 3	86 ± 7	10	80 ± 4	71 ± 2
50	87 ± 1	96 ± 1	84 ± 3	20	65 ± 4	78 ± 6	20	64 ± 2	61 ± 2
100	70 ± 4	90 ± 6	75 ± 1	35	47 ± 7	76 ± 8	40	52 ± 4	59 ± 5
200	64 ± 1	77 ± 1	67 ± 2	60	37 ± 6	62 ± 6	80	36 ± 2	—
350	49 ± 1	69 ± 3	54 ± 1	100	26 ± 5	65 ± 5			

centrations of dG up to 350 μ M, whereas RNA synthesis declined sharply between 50 and 100 μ M-dGuo (Fig. 2). The specific ¹⁴C radioactivity of the GTP pool (from [¹⁴C]Gua), measured after 4 h of incubation, slightly decreased with increasing dG concentrations in the medium. However, the decrease in specific ¹⁴C radioactivity was at most 25 %, and therefore did not account for the fall in RNA tracer incorporation. Further, the lower ¹⁴C specific radioactivity of GTP was compensated for by considering the small contribution of ³H from dG that also entered the GTP pool (by the $\sim$ 5 % residual PNP activity in dGuo-L cells). Hence dG caused a sizeable fall in RNA synthesis in dGuo-L cells, but it had no effect on DNA synthesis.

RNA synthesis in dGuo-L cell homogenates

In order to determine any effect of dGTP, and for comparison, of dATP, on RNA polymerase, the activity of this enzyme was determined in cell homogenates in the presence of the four ribonucleotides and dGTP or dATP. No consistent inhibition of RNA polymerase by dGTP or dATP was observed. Only high

concentrations of dGTP or dATP (2.5 mM) inhibited [³H]UTP incorporation by 20–30 %. Longer incubations (50 min) resulted in no effect, or even a slight stimulation of [³H]UTP incorporation by dGTP, whereas inhibition by dATP remained 30 %. On the other hand, the incorporation of [³H]GTP was decreased by 25–35 % during a 20 min incubation with 0.25 mM-dGTP. Increasing concentrations of dGTP up to 2 mM failed to cause greater inhibition.

Gel electrophoresis of cytoplasmic RNA from dGuo-L cells

Cytoplasmic RNA species were extracted with phenol/chloroform and fractionated on an agarose/urea gel. Densitometric analysis of the photographic negative showed that the amounts of 28 S rRNA, 18 S rRNA or tRNA as percentages of total cytoplasmic RNA were unchanged by dG over a 4-h incubation period (results not shown). However, 350 μ M-dG differentially depressed [³H]Gua incorporation into 28 S rRNA, 18 S rRNA and tRNA by 44 %, 46 % and 20 % respectively (Table 2).

Protein synthesis in dGuo-L cells

In dGuo-L cells, the incorporation of [³H]Leu into protein was also inhibited by dG in a dose-dependent fashion. The inhibition was more profound after longer dG exposure. For example, 100 μ M-dG caused 20, 30 and 40 % falls in tracer incorporation at 4, 8 and 16 h of incubation respectively. Comparing the time course of inhibition by dG of [³H]U and [³H]Leu incorporation into RNA and protein respectively suggested that the RNA effect was already pronounced at earlier time points (4 h), whereas the inhibition of [³H]Leu incorporation was rather small at 4 h.

Fig. 2. Effects of dG on the rates of DNA and RNA synthesis in dGuo-L cells

dGuo-L cells were incubated for 4 h with 10 μ M-mycophenolic acid and 200 μ M-Gua together with 1 μ Ci each of [¹⁴C]Gua, [³H]dG and various concentrations of dG. The rates of DNA (■) and RNA (●) synthesis are presented as the sum of the amounts of both Gua and dG incorporated (calculated on the basis of the specific radioactivity of tracers in the medium).

Table 2. Effects of dG on the incorporation of [³H]Gua into cytoplasmic RNAs

RNA	dG (μ M) ...	Incorporation of [³ H]Gua (d.p.m./ μ g)			
		0	50	100	350
28 S rRNA		1120 $\pm$ 80	780 $\pm$ 45	670 $\pm$ 40	445 $\pm$ 25
18 S rRNA		500 $\pm$ 50	445 $\pm$ 20	350 $\pm$ 10	250 $\pm$ 15
tRNA		425 $\pm$ 10	360 $\pm$ 35	340 $\pm$ 30	330 $\pm$ 10

Fig. 3. DNA analysis of the cell-cycle effect of dG on S-49 wild type (a), the PNP-negative (NSU-1, b) and the double-mutant (dGuo-L, c), cells

S-49 cells were incubated with dG, and portions of cell suspension were taken at different times. Cells were fixed with ethanol and stained with chromomycin A₃ (DNA stain) before flow-cytometric analysis. Peaks that appear before the large G₁ peak represent dead cells or cell debris with DNA content less than that of the G₁ phase cells.

Effects of dG on cell cycle of S-49 cells

After incubation with 95%-growth-inhibitory concentrations (40 μ M, 20 μ M and 350 μ M for wild-type, PNP-deficient (NSU-1) and dGuo-L cells respectively), flow cytometric analysis of total DNA content with chromomycin A₃ demonstrated a G₁-S interface blockade in the wild-type and NSU-1 cells, but not in dGuo-L cells (Fig. 3). In contrast, dGuo-L cells accumulated slightly in the G₂/M phase. Qualitatively similar changes were also observed at lower dG concentrations for all three cell lines. In order to investigate the mechanism of these changes, we performed dual-staining flow cytometry with BrdU and propidium iodide (Fig. 4). With dGuo-L cells, the progression of high-intensity green fluorescence (anti-BrdU antibody) that moved from S phase towards G₂/M phase (Fig. 4a, parts a, f-g) and finally back to G₁-phase of the cell cycle (Fig. 4a, h-i) was largely unchanged by dG as compared with the control (Fig. 4, a-e).

On the other hand, upon dG treatment of PNP-deficient mutant NSU-1 cells (Fig. 4b) and of S-49 wild-type cells (results not shown), BrdU-labelled S-phase cells continued cycling through G₂/M-phase, appeared back in G₁-phase, and were blocked at the G₁-S interface for at least 12 h (Fig. 4b, a,f-i). The slight accumulation of dGuo-L cells in G₂/M (Fig. 3) first suggested a possible M-phase blockade, since the assembly of tubulin into the mitotic apparatus requires GTP binding to tubulin subunits which might be interfered with by dGTP [28]. Quantitative analysis of the percentage of BrdU-labelled cells in each phase over time, however, gave superimposable plots of the rates of cell cycle progression. The mean T_s (DNA synthesis time [23]) for dG-treated and control dGuo-L cells was 8.09 and 8.10 h respectively. Therefore the small accumulation of the G₂/M peak did not represent a G₂/M blockade. Rather, the larger than normal G₂/M peak could result from faster cell death occurring in G₁- or S-phase cells.

DISCUSSION

We have compared the effects of dG on a double-mutant mouse T-lymphoma cell line, dGuo-L, which is PNP-deficient and has an altered RR (partially resistant to dGTP regulation),

compared with wild-type S-49 cells from which the RR hypothesis was derived [5], and with another mutant cell line, NSU-1, with PNP deficiency only.

New insights into the cytotoxic mechanism of dG by RR inhibition were gained with the combined use of tracer incorporation into DNA and analysis of cell cycle progression of S-49 wild-type and PNP-deficient (NSU-1) cells. Although dG decreased tracer incorporation into DNA, the effect was rather modest over at least the first 4 h even in the presence of toxic dG concentrations. Flow-cytometric DNA analysis revealed that the cell cycle was rapidly blocked at the G₁-S interface and the S-phase cells were progressively depleted (Fig. 3). The decreasing fraction of S-phase cells paralleled the observed progressive decrease of tracer incorporation into DNA over time. These results when combined indicate that DNA synthesis in the remaining S-phase cells was affected by dG only marginally or not at all. This conclusion was confirmed with the anti-BrdU antibody flow-cytometric analysis of cell-cycle progression: S-phase cells continued to progress through S and G₂/M into G₁-phase even in the presence of high concentrations of dG (Fig. 4). In contrast, no G₁-S interface block was observed in the double mutant, dGuo-L, and the cells cycled normally through the cell cycle, which indicated that RR inhibition was not involved in dG toxicity against the double mutant.

In view of the strong previous evidence that dG toxicity in the S-49 wild type and NSU-1 cells is associated with feedback inhibition of RR by dGTP [5], the paradoxical failure of dG to inhibit DNA synthesis in S-phase cells needed to be resolved. The dramatic differences in dGTP accumulation in G₁- and S-phase cells can resolve this paradox. G₁-phase NSU-1 and dGuo-L cells slowly and extensively accumulate dGTP (for kinetics see [25]), whereas S-phase cells show a much faster turnover rate because of ongoing DNA synthesis, and hence accumulate much less dGTP from dG. Further, we have previously shown that the direct synthesis of dGTP from dG has limited capacity relative to dGTP utilization for DNA synthesis [25]. The drastically lower dGTP accumulation in S-phase compared with G₁-phase cells is expected to mitigate dG effects specifically in S-phase cells. Therefore the lack of toxic dGTP accumulation in S-phase cells accounts for the continuing DNA synthesis in S-49 cells already in S-phase, and the increasing depression of tracer incorporation into DNA over time closely parallels the gradual depletion of S-phase cells (Figs. 3 and 4). On the other hand, the activation of RR during the G₁-S transition [26] should be blocked by pre-existing high dGTP levels, keeping the cells in G₁-phase. Hence the double mutant, dGuo-L, with impaired feedback inhibition of RR, readily passes through the G₁-S interface into S-phase, where dGTP levels rapidly drop. The continuous escape of dGuo-L cells into S-phase can therefore account for its relative resistance against dG toxicity. Furthermore, one can now propose the more general hypothesis that inhibition of RR by dGTP merely serves to keep cells in the sensitive G₁-phase where other mechanisms of dGTP/dG toxicity may be operative.

The cell cycle effects of dA, i.e. a G₁-S interface block, are very similar to those of dG. We have already documented that [³H]dA [in combination with erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA), an adenosine deaminase inhibitor] also accumulates slowly in the dATP of G₁- but not S-phase cells of the S-49 cell lines [28]. Hence dG and dA may both exert their toxicity in the G₁-phase, subsequent to a G₁-S interface blockade mediated by RR inhibition. In contrast, very different effects on cell cycle progression are observed with other agents that block RR, e.g. thymidine and hydroxyurea [29,30] which freeze cells in the S-phase. Thymidine rapidly equilibrates with the dTTP pool [27], and hydroxyurea immediately inhibits RR, thereby preventing further DNA synthesis in S-phase cells.

Fig. 4. Cell-cycle effects of dG on S-49 cells by dual-staining flow cytometric analysis

Bivariate DNA/BrdU distributions of S-49 cells were measured at different time intervals after 30 min exposure to 10 μ M-BrdU and addition of dG immediately afterwards. The intensity of both the green fluorescence (from anti-BrdU antibody, x-axis) and the red fluorescence (from propidium iodide, y-axis) is expressed on a logarithmic scale. (a) Progression of control (a-e) and dG (350 μ M)-treated (f-i) dGuo-L cells through the cell cycle. (b) Cell cycle of control (a-e) and dG (20 μ M)-treated (f-i) PNP negative mutant S49 cells. Parallel results obtained with S-49 wild-type cells were identical to those of the PNP-negative mutant.

Our extensive studies with several tracers to measure RNA synthesis clearly indicate an early inhibition of RNA synthesis by dG, followed by an inhibition of [3 H]Leu incorporation into proteins. The mechanisms by which dG affects RNA synthesis and metabolism, as shown in this study, remain to be investigated

in detail, but they appear to be unrelated to competition between dGTP and GTP for RNA polymerases.

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