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2′-Deoxycoformycin Toxicity in Murine Spleen Lymphocytes

MARTIN F. EARLE AND ROBERT I. GLAZER

Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

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SUMMARY

To investigate the direct lymphocytic toxicity of the adenosine deaminase inhibitor, 2′-deoxycoformycin (dCF), an *in vitro* model system employing concanavalin A-stimulated mouse spleen lymphocytes was utilized. When lymphocytes were incubated for 24 or 66 hr with 10^{-8} M–10^{-4} M dCF added 30 min prior to addition of mitogen, a time- and dose-dependent inhibition of DNA and RNA synthesis occurred. The IC_{50} values for DNA and RNA synthesis and adenosine deaminase activity 66 hr after drug exposure were 10^{-6} M, 3×10^{-6} M, and 3×10^{-9} M, respectively. When lymphoblastogenesis was measured 24 hr after the addition of 10^{-4} M dCF, DNA and RNA syntheses were inhibited 10% and 30%, respectively, despite the same marked inhibition of adenosine deaminase activity observed at 66 hr. Following 66 hr of treatment with 10^{-6} dCF, methylation of RNA and DNA were not affected, but a moderately higher degree of DNA but not RNA methylation was noted following exposure to 10^{-4} M dCF. Measurement of cellular ribonucleotide levels 24 and 66 hr after drug exposure revealed a dose-dependent reduction in nucleoside mono- and triphosphates. In contrast, a 2- to 2.5-fold elevation in IMP levels at 10^{-6} M dCF and 50%–100% reduction in IMP levels at 10^{-4} M dCF were observed at both 24 and 66 hr after treatment. However, levels of dATP were not significantly altered 24 and 66 hr after treatment with dCF. These data suggest that inhibition of nucleic acid synthesis by dCF is not dose-related to the inhibition of adenosine deaminase, that DNA and RNA methylation as well as dATP levels are unaffected, and that changes in nucleotide levels accompany drug-mediated lymphocytic toxicity.

INTRODUCTION

dCF[1] is a bacterial fermentation product of *Streptomyces antibioticus* and is a potent, tightly binding, transition-state inhibitor of adenosine deaminase (EC 3.5.4.4) (1). When administered to mice, it was found to produce profound immunosuppression manifested by peripheral lymphopenia, involution of the lymphoid organs (2), and the acceptance of mouse skin allografts across major histocompatibility barriers (3). In humans, the drug has been shown to produce lymphopenia (4) and has antitumor activity against acute lymphoblastic leukemia (5, 6). chronic lymphocytic leukemia (7), and mycosis fungoides (6).

In vitro studies concerning the mechanism of action of dCF have been limited by the lack of a suitable tumor cell line exhibiting toxicity to the drug. Potentiation of AR (8) or AdR (9) toxicity by adenosine deaminase inhibitors in mitogen-stimulated lymphocytes has led to the speculation that dCF produces cellular toxocity through the intracellular buildup of AR, AdR, and/or their metabolites. *In vitro* incubations of mitogen-stimulated murine or human lymphocytes with dCF alone have not produced consistent antiproliferative effects (10–12); however, Chassin *et al.* (13) showed that long-term incubation of mitogen-stimulated mouse and human lymphocytes with dCF resulted in a dose-dependent reduction of DNA synthesis with an IC_{50} of 10^{-6} M.

It has been assumed that the toxicity imparted by dCF is mediated by its inhibition of adenosine deaminease; however, no studies have clearly correlated the inhibition of lymphocyte proliferation with the inhibition of adenosine deaminase in intact cells. In this report we demonstrate that dCF-induced inhibition of blastogenesis in mouse spleen lymphocytes does not appear to be causally related to inhibition of adenosine deaminase, nucleic acid methylation, or dATP levels, but is associated with reductions in cellular ribonucleotide concentrations.

EXPERIMENTAL PROCEDURES

Materials. dCF was obtained from the Drug Synthesis and Development Branch, National Cancer Institute. [*methyl*-^{6}H]Methionine (80 Ci/mmole), [*methyl*-^{3}H]TdR (20 Ci/mmole), [5-^{3}H]UR (25.5 Ci/mmole), [U-^{14}C]UR (522 mCi/mmole), [2-^{14}C]CdR (29.7 mCi/mole), and

[1] The abbreviations used are: dCF, 2′-deoxycoformycin; AR, adenosine; AdR, deoxyadenosine; TdR, thymidine; UR, uridine; IR, inosine; m^5CdR, 5-methyldeoxycytidine; conA, concanavalin A; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline (6.6 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 0.154 M NaCl, pH 7.4.

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[*methyl*-^{3}H]dTTP (18.8 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). Ficoll-Paque was obtained from Pharmacia (Piscataway, N. J.). ConA was obtained from Calbiochem (La Jolla, Calif.). AR, IR, RNase A (Type X-A), RNase T_1 (Grade III), and alkaline phosphatase (Type III-R) were obtained from Sigma Chemical Company (St. Louis, Mo.); snake venom phosphodiesterase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); DNase I and *Micrococcus luteus* DNA polymerase were obtained from Miles Laboratories (Elkhart, Ind.); poly(dA-dT)·poly(dA-dT) was obtained from P-L Biochemicals (Milwaukee, Wisc.).

Isolation of lymphocytes. Female Swiss mice weighing 20–25 g were killed by cervical dislocation. Their spleens were removed aseptically and teased apart in a 10-cm Petri dish containing RPMI medium 1640 supplemented with 15% newborn calf serum and gentamicin, 75 μg/ml. The cells were incubated for 2 hr in 5% CO_2/air at 37° to allow macrophage adherence to the plastic surface of the dish. Cells were then filtered through sterile gauze to remove debris, layered over Ficoll-Paque, and centrifuged at 300 × *g* for 30 min at room temperature. Lymphocytes were collected at the Ficoll-Paque-media interface, washed with fresh media three times, and resuspended in fresh media.

Measurement of lymphocyte blastogenesis. Lymphocytes were incubated at 37° under an atmosphere of 5% CO_2/air in microtiter plates (Costar, Cambridge, Mass.) containing RPMI medium 1640 supplemented with 15% dialyzed heat-inactivated newborn calf serum and gentamicin, 75 μg/ml. Each well contained 2.5×10^5 cells in a final volume of 0.2 ml. Lymphocytes were preincubated for 30 min with 10^{-9} to 10^{-4} M dCF prior to addition of mitogen (conA, 5 μg/ml) unless specified otherwise. dCF treatment was carried out with conA-stimulated lymphocytes in all instances. At 20 and 62 hr after addition of mitogen, 1 μCi of [^{3}H]TdR or 1 μCi of [^{3}H]UR was added and incubation was continued for an additional 4 hr. Cells were harvested onto glass-fiber filter paper and washed with 5% perchloric acid; radioactivity was determined in a Searle Mark III liquid scintillation counter.

Adenosine deaminase assay. Control and dCF-treated lymphocytes were assayed for adenosine deaminase activity by a modification of the method by Hartwick *et al.* (14) concurrent with the measurement of [^{3}H]TdR and [^{3}H]UR incorporation. Cell-free extracts of lymphocytes were obtained by lysis in water at 4°. AR was added to the extract at a final concentration of 2.5 mM, and the final assay volume was adjusted to 1.0 ml with PBS and incubated at 37° for 1 hr. The reaction was stopped by the addition of 1 N HCl. The formation of IR was measured by reverse-phase HPLC utilizing 0.01 M KH_2PO_4 (pH 3.8)/14% methanol at a flow rate of 1.5 ml/min. Peak areas were measured by a Hewlett Packard 3390A integrator and compared with standards.

Methylation of RNA and DNA. m^{5}CdR levels in DNA were measured in control and dCF-treated lymphocytes following incubation of 10^8 cells in 40 ml of media for 66 hr. The washed cell pellets were extracted with 3 ml of 1% SDS/0.1 M Tris-HCl (pH 8.0)/0.1 M EDTA and vortexed for 1 min. The extract was mixed with 1.5 ml of a mixture of phenol/*m*-cresol/water (7:2:2, v/v) containing 0.1% 8-hydroxyquinoline followed by 1.5 ml of chloroform. Samples were centrifuged at 12,000 × *g* at 4° for 10 min. The upper aqueous layer containing the RNA and DNA was removed and precipitated with 3 volumes of 2% potassium acetate dissolved in 95% ethanol at −20° overnight. Samples were then centrifuged at 12,000 × *g* at 4° for 20 min. The precipitated RNA and DNA were washed with 2% potassium acetate in 95% ethanol solution and dissolved in 1 ml of 0.01 M Tris-HCl (pH 7.4)/0.2 M NaCl/0.01 M EDTA. The RNA was digested with 0.01 ml of RNase A (2 mg/ml) and 0.01 ml of RNase T_1 (200 units/ml) for 2 hr at 37°. The remaining DNA was precipitated with 3 ml of 2% potassium acetate in 95% ethanol at −20° overnight. The resulting DNA precipitates were washed with 2% potassium acetate in 95% ethanol at −20° and dissolved in 1 ml of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/5 mM $MgCl_2$ (pH 7.5). The DNA was digested with 20 μl/DNase I (1 mg/ml), 10 μl of snake venom phosophodiesterase (1.5 units/ml), and 10 μl of alkaline phosphatase (421.2 units/ml) for 1 hr at 37°. The resulting solutions were lyophilized and redissolved in 0.2 ml of water, and deoxyribonucleosides were separated by HPLC utilizing a Brownlee RP-18 Spheri-5 5-μm column (0.4 × 10 cm) and 0.01 M KH_2PO_4/5% methanol at a flow rate of 1.5 ml/min.

The rate of methylation of DNA was measured in lymphocytes treated with 10^{-6} M and 10^{-4} M dCF for 66 hr by pulse labeling for 4 hr with 500 μCi of [*methyl*-^{3}H] methionine and 2 μCi of [^{14}C]TdR prior to harvesting the cells. The washed cell pellets were extracted as described above and the radioactivity in DNA was determined.

The rate of RNA methylation was measured in lymphocytes treated with 10^{-6} M and 10^{-4} M dCf for 24 hr by pulse-labeling for 4 hr with 500 μCi of [*methyl*-^{3}H]methionine and 2 μCi of [U^{14}C]UR. The washed cell pellets were extracted with 1% SDS buffer and precipitated as described above. The isolated nucleic acids were dissolved in 1 ml of 0.01 M Tris-HCl (pH 7.2)/0.5 M NaCl/0.01 M $MgCl_2$ to which 20 μl of a 1 mg/ml solution of RNase-free DNase I was added, and incubated for 2 hr at 37°. The RNA was precipitated with 3 volumes of 2% potassium acetate in 95% ethanol at −20° overnight, and washed with 95% ethanol.

Nucleotide analyses. Control and dCF-treated lymphocytes (10^8 cells) were incubated in 40 ml of media for 24 or 66 hr. Cells were harvested, washed with cold PBS, and centrifuged at 300 × *g*. Cell pellets were extracted with 0.2 ml of cold 5% trichloroacetic acid and centrifuged at 16,000 × *g* in an Eppendorf centrifuge; the supernatants were neutralized with 2 volumes of 0.5 M trioctylamine in trifluorotrichloroethane (15) by stirring on a Vortex mixer for 10 min. Samples were centrifuged at 16,000 × *g*, and supernatant fractions were analyzed by anion-exchange HPLC utilizing a Brownlee amino Spheri-5 column (0.4 × 10 cm). Nucleoside monophosphates were eluted isocratically at 2 ml/min for 10 min with 0.0135 M KH_2PO_4, (pH 3.7) followed by a linear gradient over 20 min to 0.3 M KH_2PO_4/0.3 M KCl (pH 3.8), which was then continued isocratically until all nucleoside triphosphates were eluted. Peak areas were measured with a

Hewlett Packard 3390A integrator and were compared with standards.

AdR measurement. Lymphocytes were incubated for 24 or 66 hr with 10^{-7} to 10^{-4} M dCF. Cells were harvested by centrifugation at 300 × *g*, and the cell pellets were washed with cold PBS and saved for dATP determinations as described below. The supernatant fractions were centrifuged at 750 × *g* through Amicon Centriflo (Danvers, Mass.) membrane cones (Type CF 25). Ultrafiltrates were lyophilized and dissolved in 0.2 ml of water. AdR was measured by reverse-phase HPLC utilizing a Brownlee RP-18 Spheri-5 5-μm column and 0.01 M KH_2PO_4 (pH 3.8)/5% methanol at a flow rate of 2 ml/min. Peaks were integrated as previously described and were compared with standards.

dATP analyses. dATP concentrations were measured in neutralized (15) 5% trichloroacetic acid extracts of the cell pellets obtained as described above, by the DNA polymerase assay of Hunting and Henderson (16), using a 30-min assay. dATP standards (2.5–40 pmoles) treated in a manner similar to that employed for cell extracts produced a linear standardization curve under these assay conditions.

RESULTS

In conA-stimulated mouse spleen lymphocytes, total RNA synthesis as measured by [^{3}H]UR incorporation reached a peak at 24 hr after mitogen stimulation and began to decline after 48 hr. DNA synthesis, as measured by [^{3}H]TdR incorporation, began at 24 hr and reached a maximum at 66 hr after mitogen stimulation. Because of these observations, experiments were carried out at 24 hr and 66 hr after conA stimulation in most of the subsequent experiments.

Time dependence of dCF exposure. Initial experiments were carried out to determine the schedule dependency between the time of addition of dCF and conA with respect to the degree of lymphocyte blastogenesis as monitored by [^{3}H]TdR incorporation (Fig. 1). When dCF was added to the cultures 1 hr prior to mitogen stimulation, 88% and 72% inhibition of [^{3}H]TdR incorporation into DNA occurred at 10^{-4} M and 10^{-6} M dCF, respectively. The addition of dCF after mitogen produced a progressively lesser inhibition of blastogenesis. Upon the addition of dCF 66 hr after conA, little or no inhibition of blastogenesis occurred. In all subsequent experiments, dCF was added 30 min prior to the addition of conA.

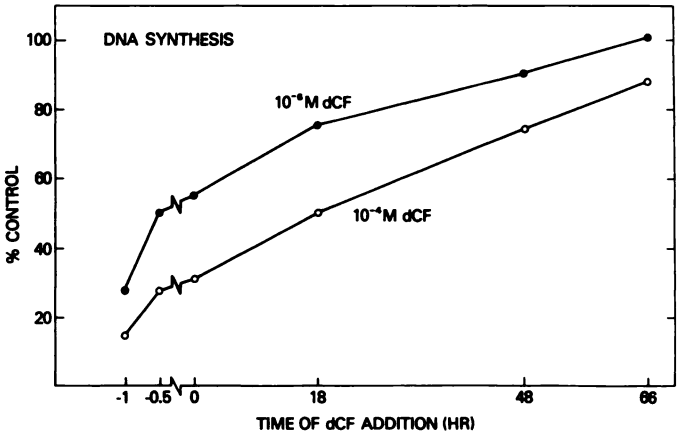

FIG. 1. *[^{3}H]TdR incorporation into DNA versus the exposure interval to dCF*

Time 0 represents the time of conA addition to the lymphocytes. Negative numbers represent the time at which dCF was added to the cultures prior to conA addition. All cells were harvested at 66 hr as described under Experimental Procedures. The range of values (disintegrations per minute of [^{3}H]TdR incorporated per 5 × 10^5 cells/4 hr) for controls was 102,500–176,400.

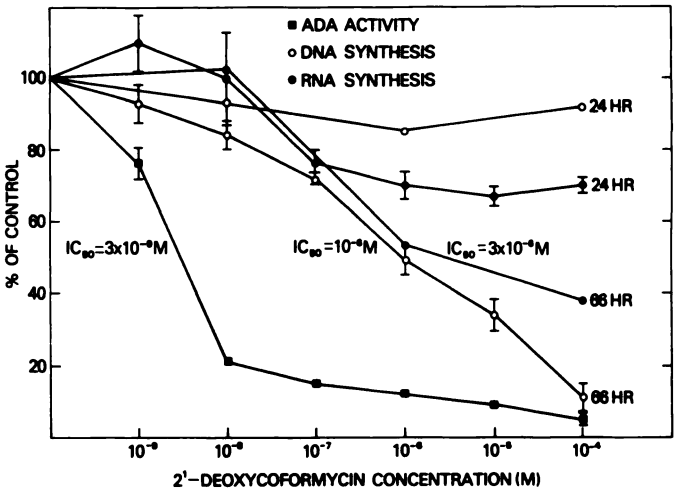

FIG. 2. *[^{3}H]TdR and [^{3}H]UR incorporation into DNA and RNA and adenosine deaminase (ADA) activity of lymphocytes after dCF treatment*

DNA and RNA syntheses were measured 24 hr and 66 hr after drug exposure whereas adenosine deaminase activity was measured at 66 hr as described under Experimental Procedures. The means or means ± standard error for control values (disintegration per minute incorporated per 5 × 10^5 cells/4 hr) were as follows: [^{3}H]UR at 24 hr, 37,000 ± 4500; [^{3}H]TdR at 24 hr, 5300; [^{3}H]UR at 66 hr, 24,100; [^{3}H]TdR at 66 hr, 146,500 ± 16,700. The mean ± standard error for adenosine deaminase activity (nanomoles of IR produced per 5 × 10^5 cells/hr) in controls was 10.2 ± 1.1.

Inhibition of adenosine deaminase activity and lymphocyte blastogenesis. Since dCF appeared to exert a specific antiblastogenic effect when added prior to conA, a dose-response study was performed to characterize this phenomenon on both RNA and DNA synthesis and adenosine deaminase activity. The inhibition of adenosine deaminase by dCF in lymphocytes 66 hr after stimulation with conA and the inhibition of [^{3}H]TdR and [^{3}H]UR incorporation into nucleic acids at 24 and 66 hr after mitogen stimulation are shown in Fig. 2. The inhibition of adenosine deaminase 24 hr after drug treatment (data not shown) was identical with that at 66 hr and demonstrated a dose responsiveness with an IC_{50} of 3 × 10^{-9} M. At 24 hr after addition of dCF, there was no inhibition of [^{3}H]TdR incorporation; however, there was a 20–30% inhibition of [^{3}H]UR incorporation at 10^{-7} M dCF but without further inhibition at higher dCF concentrations. At 66 hr after addition of dCF, a dose-dependent reduction of [^{3}H]TdR and [^{3}H]UR incorporation by dCF occurred where the IC_{50} was 10^{-6} M and 3 × 10^{-6} M, respectively.

Measurement of DNA and RNA methylation. Reverse-phase HPLC analysis of DNA digests of lymphocytes treated for 66 hr with dCF revealed no change in

TABLE 1

Nucleic acid methylation in conA-stimulated lymphocytes treated with dCF

Lymphocytes were treated for 24 hr (RNA methylation) or 66 hr (DNA methylation), and RNA and DNA methylation was measured by pulse-labeling with [*methyl*-^{3}H]methionine plus [^{14}C]UR or [*methyl*-^{3}H]methionine plus [^{14}C]TdR, respectively, as described under Experimental Procedures.

Treatment	DNA			RNA		
	^{3}H	^{14}C	^{3}H/^{14}C	^{3}H	^{14}C	^{3}H/^{14}C
	dpm/A$_{260}$			*dpm/A*$_{260}$		
Control	4,150	27,440	0.15	75,380	67,080	1.12
dCF, 10^{-6} M	3,450	19,890	0.17	71,760	59,790	1.20
dCF, 10^{-4} M	4,400	17,830	0.25	61,660	55,570	1.11

the percentage of m^{5}CdR (data not shown). Control cells and those treated with 10^{-6} M or 10^{-4} M dCF contained 7.7%, 8.1%, and 7.9% m^{5}CdR, respectively.

The rates of DNA and RNA methylation were measured by pulse-labeling cells for 4 hr with either [^{3}H]methionine and [^{14}C]TdR or with [^{3}H]methionine and [^{14}C]UR, respectively (Table 1). The ratio of [^{3}H]methionine/[^{14}C]UR in RNA or [^{3}H]methionine/[^{14}C]TdR in DNA did not appreciably change following treatment of lymphocytes with 10^{-6} or 10^{-4} M dCF for 24 hr (RNA) or with 10^{-6} M dCF for 66 hr (DNA); however, a 67% increase in methylation of DNA by 10^{-4} M dCF was noted.

Measurement of nucleotide levels. The results of anion-exchange HPLC analyses of nucleoside mono- and triphosphate levels in dCF-treated lymphocytes 24 and 66 hr after treatment are shown in Table 2. At 24 hr, AMP, GMP, and UMP levels were reduced 12–28% by 10^{-6} M dCF, and 20–46% by 10^{-4} M dCF. IMP levels gave a biphasic response to dCF treatment whereby 10^{-6} M dCF produced a 247% increase, and 10^{-4} M dCF a 49% decrease. Nucleoside triphosphates at 24 hr showed little change except at 10^{-4} M dCF, where ATP increased 28% and GTP decreased 50%. With 66-hr exposure to dCF, more profound changes in mono- and triphosphate nucleotides occurred. AMP, GMP, and UMP levels were reduced 42–49% by 10^{-6} dCF and 53–89% by 10^{-4} M dCF. IMP levels were increased by 215% by 10^{-6} dCF but were decreased to nondetectable levels by 10^{-4} M dCF. ATP, GTP, UTP, and CTP levels 66 hr after dCF treatment were reduced 21–42% by 10^{-6} M dCF and by 44–73% by 10^{-4} M dCF.

TABLE 2

Nucleotide levels in conA-stimulated lymphocytes treated with dCF

Lymphocytes were treated for 24 or 66 hr with 10^{-6} and 10^{-4} M dCF, and nucleotides were measured as described under Experimental Procedures. Each value represents the mean ± standard error of three experiments.

Nucleotide	dCF concentration			
	24-hr exposure		66-hr exposure	
	10^{-6} M	10^{-4} M	10^{-6} M	10^{-4} M
	% Control[a]			
AMP	83 ± 8	71 ± 5	51 ± 9	47 ± 6
UMP	88 ± 10	80 ± 7	58 ± 2	11 ± 6
IMP	247 ± 26	51 ± 12	215 ± 45	ND[b]
GMP	72 ± 10	54 ± 10	51 ± 8	25 ± 3
CTP	92 ± 6	78 ± 5	79 ± 2	39 ± 7
UTP	85 ± 5	82 ± 5	75 ± 3	33 ± 2
ATP	87 ± 4	128 ± 7	58 ± 4	56 ± 3
GTP	89 ± 3	50 ± 2	73 ± 5	27 ± 5

[a] The mean 24-hr control values (nanomoles per 10^8 cells) were as follows: AMP, 1.87; UMP, 0.50; IMP, 3.98; GMP, 0.26; CTP, 0.80; UTP, 4.64; ATP, 24.00; GTP, 3.31. The mean 66-hr control values were as follows: AMP, 3.02; UMP, 1.19; IMP, 0.72; GMP, 0.41; CTP, 2.95; UTP, 8.64; ATP, 28.32; GTP, 5.89.

[b] None detected.

TABLE 3

dATP levels in conA-stimulated lymphocytes treated with dCF

Lymphocytes were treated for 24 or 66 hr with 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M dCF, and dATP levels were measured as described under Experimental Procedures. Each value represents the mean ± standard error of three experiments.

dCF concentration	Duration of drug exposure	dATP concentration[a]
M	*hr*	*% Control*
10^{-7}	24	109 ± 4
10^{-6}	24	103 ± 6
10^{-5}	24	91 ± 2
10^{-4}	24	87 ± 4
10^{-7}	66	120 ± 2
10^{-6}	66	119 ± 3
10^{-5}	66	129 ± 6
10^{-4}	66	138 ± 8

[a] The mean 24-hr control value (picomoles of dATP per 10^8 cells) was 151 ± 6, and the mean 66-hr control value was 100 ± 5.

AdR was not detectable in the media of untreated control cultures; however, the media of lymphocytes treated for 24 to 66 hr with 10^{-7} to 10^{-4} M dCF contained approximately 1 μM AdR (results not shown). This concentration of AdR was independent of dCF concentration and appeared to reflect inhibition of adenosine deaminase in the media by dCF. No AR could be detected in the media of treated or untreated cultures.

Lymphocyte dATP levels following exposure to 10^{-7} to 10^{-4} M dCF revealed no significant changes after 24 hr of drug treatment and only modest increases after 66 hr of exposure to dCF (Table 3).

DISCUSSION

The present study confirms in a model system of conA-stimulated mouse spleen lymphocytes (13) that inhibition of blastogenesis can be produced by long-term exposure to dCF. Inhibition of blastogenesis clearly correlated with drug exposure time, and drug exposure prior to mitogen stimulation markedly enhanced toxicity.

The inhibition of lymphocyte blastogenesis by dCF in previously published reports is controversial. Burridge *et al.* (10) demonstrated no inhibition of the incorporation of [^{3}H]TdR by 3.75×10^{-6} M dCF in conA-stimulated spleen cells taken from mice who were additionally treated *in vivo* with dCF at a dose of 1.0 mg/kg daily for 4 days. Trotta *et al.* (11) treated mice with dCF by continuous infusion at a dose of 0.4 mg/kg/day for 5 days, and found that this regimen produced 66–73%

inhibition of [^{3}H]TdR incorporation in conA-stimulated mouse spleen cells incubated *in vitro*. Uberti *et al.* (12) showed that in conA-stimulated human lymphocytes incubated *in vitro* with 10^{-6} dCF, the incorporation of [^{3}H]leucine was inhibited by only 16%. Chassin *et al.* (13) reported inhibition of the incorporation of [^{3}H]TdR in conA-stimulated mouse spleen lymphocytes by preincubation of the cells with dCF prior to mitogen stimulation. The dose-responsive inhibition of lymphocyte blastogenesis by dCF with an IC_{50} of 10^{-6} M is confirmed by our study. The variability in the reported toxicity by dCF is not clear. Mouse spleen lymphocytes as well as human peripheral lymphocytes are heterogeneous populations of cells. Experiments in mouse spleen cells by Ballow and Pantschenko (17) suggest that there are two subpopulations of mitogen-responsive T cells, one which is sensitive and the other which is relatively resistant to the inhibitory effects of dCF. Variability in the ratio of these subpopulations could, in theory, account for the inconsistent inhibition of blastogenesis by dCF.

A lack of correlation between the inhibition of lymphocyte blastogenesis by dCF and the inhibition of lymphocyte adenosine deaminase activity has been suggested (10, 16). The 50% inhibition of [^{3}H]TdR and [^{3}H]UR incorporation 66 hr after drug exposure requires a dCF concentration that is 333 and 1000 times greater, respectively, than that necessary to inhibit adenosine deaminase in whole cells. Similarly, 24 hr after drug treatment under identical conditions of adenosine deaminase inhibition, no inhibition of [^{3}H]TdR incorporation and only 30% inhibition of [^{3}H]UR occurred. Certainly this suggests mechanisms other then adenosine deaminase by which dCF inhibits lymphocyte blastogenesis.

Our measurements of m^{5}CdR levels in DNA and the rate of DNA and RNA methylation in dCF-treated lymphocytes indicate that this drug does not appreciably affect the methylation of nucleic acids. The method employed to measure the rate of methylation used high concentrations of methionine in the medium, rendering it unlikely that nonspecific alterations in the concentration of this amino acid would lead to spurious results. The lack of inhibition of methylation, in spite of AdR levels of approximately 1 μM in the media of dCF-treated lymphocytes, is quite consistent with reports in the literature. Glazer and Hartman (18) demonstrated that neither dCF alone nor dCF plus 250 μM AdR affected the methylation of nuclear RNA in L1210 cells. Hershfield and Kredich (19) also demonstrated that as little as 20 μM AdR inhibited *S*-adenosylhomocysteine hydrolase by 80% within 24 hr, but that these conditions did not result in significant accumulations of *S*-adenosylhomocysteine or dATP or inhibition of growth of human T lymphoblastoid cells. The significance of the higher rate of methylation of only DNA by 10^{-4} M dCF (Table 1) is not understood. Since hypermethylation is generally associated with reduced transcription (20), this effect may have relevance to inhibition of blastogenesis.

That inhibition of ribonucleotide reductase by accumulation of dATP is not playing a significant role in dCF-induced toxicity is suggested by the data showing that dCF did not produce a significant increase in dATP levels. This is consistent with the finding that mouse spleen lymphocytes in the presence of AdR produce only small elevations of dATP as compared with human lymphocytes (21). The presence of 1 μM AdR also produces only modest elevations of cellular dATP in T and non-T lymphocytes (22).

The most significant change corresponding to the inhibition of lymphocyte blastogenesis was a dose-dependent reduction in ribonucleotide concentrations. The lack of significant inhibition of RNA and DNA synthesis 24 hr after dCF treatment and the dose-dependent reduction in RNA and DNA synthesis 66 hr after drug exposure correlated relatively well with the reductions in the levels of AMP, GMP, UMP, GTP, UTP, and CTP. Of interest was the biphasic change in the concentration of IMP at both 24 hr and 66 hr after treatment. This may possibly be explained by inhibition of adenosine deaminase and adenylate deaminase at different concentrations of dCF. dCF is a noncompetitive inhibitor of rabbit muscle adenylate deaminase, with a k_i of 3×10^{-6} M (23). In lymphocyte extracts, dCF competitively inhibited AMP deaminase with an IC_{50} of 10^{-4} M at 0.1 mM AMP (data not shown). Because of the limitation of intracellular transport by the nucleoside transport system (24, 25), one can postulate that at 10^{-6} M dCF, adenosine deaminase is completely inhibited while AMP deaminase is not. If IMP utilization is inhibited, one can expect IMP to increase with treatment. However, at higher drug concentrations, AMP deaminase may be inhibited through an accumulation of intracellular dCF and/or a metabolite of dCF. These effects would be expected to lead to increases in the levels of AMP with simultaneous decreases in the concentration of IMP.

Data indicating changes in cellular nucleotide levels after treatment with dCF was presented by Henderson *et al.* (26), who showed that dCF inhibited purine ribonucleotide biosynthesis via the conversion of IMP to AMP and GMP in Ehrlich ascites cells. The dose- and time-dependent reductions in GTP may in turn play an important role in inhibiting DNA synthesis. This is supported by the work of Cohen *et al.* (27), who demonstrated that reductions in GTP rather than dGTP were important in inhibiting DNA synthesis in mycophenolic acid-treated S49 lymphoma cells. Alternatively, it is plausible that inhibition of [^{3}H]TdR incorporation by dCF is secondary to inhibition of RNA synthesis or some other event which produces a prolongation in the cell cycle of mitogen-activated lymphocytes.

The reductions in pyrimidine nucleotide levels also appeared to be dose- and time-dependent. The mechanism by which this reduction occurs is unknown. Inhibition of 5-phosphoribosyl-1-pyrophosphate synthesis in lymphoid tissues occurred after infusion of dCF at 0.4 mg/kg/day for 5 days (28); however, Henderson *et al.* (26) failed to observe inhibition of *de novo* purine synthesis *in vitro* by 185 μM dCF, a pathway also dependent on 5-phosphoribosyl-1-pyrophosphate. Because of reductions in the measured uridine nucleotides in treated cells, the intracellular specific radioactivity of [^{3}H]UTP likely increased with increasing dCF concentration, thereby blunting the measured inhibition of [^{3}H]UR incorporation. This effect may also hold for the specific activity of [^{3}H]dTTP, which was not directly measured.

We conclude that the action of dCF on conA-stimulated mouse spleen cells appears to be related to the time of drug exposure, is not solely dose-related to adenosine deaminase inhibition, does not involve inhibition of DNA or RNA methylation, is not associated with dATP accumulation, but is accompanied by reductions in ribonucleotide concentrations.

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Send reprint requests to: Dr. Robert I. Glazer, Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Building 37, Room 6D28, Bethesda, Md. 20205.