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Effects of Dicyclohexylamine Sulfate, a Spermidine Synthase Inhibitor, in 9L Rat Brain Tumor Cells[1]

Burt G. Feuerstein, Dennis F. Deen, and Laurence J. Marton[2]

Brain Tumor Research Center of the Department of Neurological Surgery [B. G. F., D. F. D., L. J. M.] and the Departments of Radiation Oncology [D. F. D.] and Laboratory Medicine [L. J. M.], School of Medicine, University of California, San Francisco, California 94143

ABSTRACT

Growth characteristics, polyamine levels, and distribution of cells in the cell cycle were determined for 9L rat brain tumor cells treated for various periods with 1 mM dicyclohexylamine sulfate (DCHA). Continuous treatment of cells with DCHA caused growth inhibition at 2 days of treatment. After 2 days of treatment the growth rate of cells increased to approximately the same rate as control cells, even though treatment was continuous. Levels of spermidine were depleted to less than 10% of control levels, spermine levels were essentially unchanged, and putrescine levels were elevated to more than 350% of control levels after 9L cells were treated with DCHA for 2 days. In contrast to results found for the polyamine biosynthesis inhibitor α-difluoromethylornithine, treatment of 9L cells with DCHA did not potentiate the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea. To mimic the effects on polyamine levels caused by treatment with DCHA, 9L cells were treated with 5 mM putrescine alone or with 5 mM putrescine and 1 mM DCHA after treatment with 1 mM α-difluoromethylornithine. Results of these experiments suggest that treatment with DCHA alone does not potentiate the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea because elevated levels of putrescine caused by treatment counteract the effects of decreased spermidine levels.

INTRODUCTION

We have reported that polyamine depletion induced by two polyamine biosynthesis inhibitors, MGBG[3] and DFMO, alters the cytotoxic effects of several DNA directed anticancer agents (1–4). The agent dependent increase or decrease in cytotoxicity can be prevented by addition of polyamines (1–4). A detailed study of the potentiation of BCNU cytotoxicity by DFMO pretreatment showed a temporal relationship with polyamine depletion and a cytotoxic dose response that was similar to that of polyamine depletion (5).

It is well known that polyamines interact with nucleic acids. SP is important for the crystallization of both the Z and B forms of DNA and appears to bridge the major groove of the B form (6). Polyamines are known to condense DNA (7), to stabilize the DNA helix to thermal melting (8, 9), and to promote the Z to B transition in synthetic heteropolymers (10).

We have argued that alterations in intracellular levels of polyamines and the effect on DNA conformation are the primary cause of the modification of cytotoxicity of DNA directed agents. This hypothesis is supported by several lines of evidence. We have shown that the viscoelastic response of DNA, and thus its conformation, is altered by DFMO treatment (11). DFMO pretreatment alters the expression of sister chromatid exchange (12), formation of cross-links (13), and cytotoxicity of *cis*-platinum and BCNU (1, 4). Uniformly these effects can be prevented by the addition of exogenous PU.

We have investigated the effects of DCHA, an inhibitor of SD synthase (14), on polyamine levels, growth, cell cycle distribution, and cytotoxicity of BCNU in 9L cells. The results suggest that in 9L cells the levels of PU produced by treatment with DCHA are sufficiently elevated that PU can substitute for the function(s) of SD and that potentiation of the cytotoxicity of BCNU is partially prevented.

MATERIALS AND METHODS

Drugs. DFMO was generously supplied by the Merrell-Dow Research Institute (Cincinnati, OH). DCHA was obtained from the Sigma Chemical Co. (St. Louis, MO), and PU and chromomycin A_3 were supplied by Calbiochem-Boehringer Corp. (La Jolla, CA). BCNU was provided by the National Cancer Institute (Bethesda, MD).

Cell Culture. Monolayer cultures of 9L cells were maintained at 37°C in a 95% air-5% CO_2 atmosphere in MEM supplemented with nonessential amino acids, gentamicin (50 μg/ml), and 5% Nu Serum (Collaborative Research, Lexington, MA). Cells were washed and incubated at 37°C for 5 to 10 min in saline-trypsin-Versene. To ensure the presence of single cell suspensions after incubation in saline-trypsin-Versene, flasks were rapped against the laboratory bench and the cells were pipeted five to eight times and kept on ice before reseeding.

Polyamine Depletion. Cells were grown for 24 h before treatment with DCHA or DFMO. Stock solutions (300 mM) of DCHA and DFMO were prepared in MEM without additives (pH 7.5). After dilution of the appropriate volume of stock solution into cell culture medium, cells were treated with 1 mM DCHA or DFMO for at least 48 h.

PU alone was replenished in DFMO treated cells by treating with 1 mM DCHA for 1 h (to inhibit metabolism of PU to SD), after which 5 mM PU was added to cultures for 1 h. In some experiments cells were not treated with DCHA before addition of exogenous PU to medium. After PU was added using either protocol, cells were treated for 1 h with several concentrations of BCNU (Chart 1).

Cell Growth and Polyamine Levels. Cells were seeded in separate duplicate flasks for each time point and treated with either DCHA or DFMO. At the appropriate time cells were harvested and counted electronically. Data represent the average of two duplicate flasks. The polyamine assay has been described (15).

BCNU Treatment. Stock solutions of BCNU (10 mM) were prepared in absolute ethanol immediately before use. The final concentration of ethanol in MEM did not affect plating efficiency.

Cell Survival Assay. This assay has been described (16). Briefly Petri dishes were seeded with 5×10^4 heavily irradiated (40 Gy) 9L feeder

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[2] To whom requests for reprints should be addressed, at the Department of Laboratory Medicine, L518, University of California, San Francisco, CA 94143.

[3] The abbreviations used are: MGBG, methylglyoxal-bis(guanylhydrazone); DFMO, α-difluoromethylornithine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; SP, spermine; PU, putrescine; DCHA, dicyclohexylammonium sulfate; SD, spermidine; MEM, Eagle's minimum essential medium; FCM, flow cytometry.

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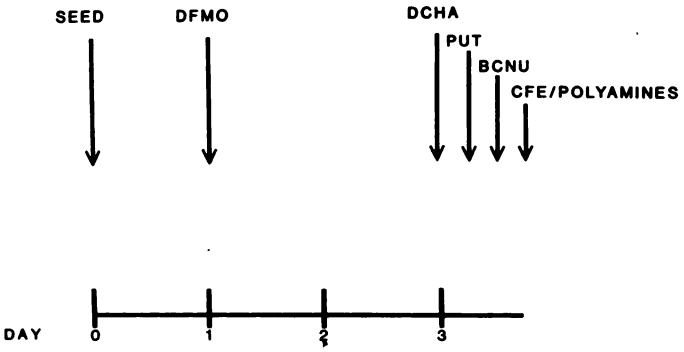

Chart 1. Design for PU addition experiment. CFE, colony forming efficiency assay.

cells in 4 ml of MEM. After drug treatment an appropriate number of cells were seeded into Petri dishes and incubated at 37°C for 12 to 14 days. Colonies with more than 50 cells were scored. The surviving fraction of BCNU treated cells was normalized for the cell kill caused by DCHA by subtracting the number of colonies that grew after DCHA pretreatment from the number of colonies that grew after BCNU treatment.

FCM. 9L cells were collected in single cell suspension after treatment, centrifuged at 500 rpm for 5 min, and resuspended in 70% ethanol, after which they were stained with chromomycin A_3 for at least 30 min and analyzed on a FACS II (Becton-Dickinson, Mountain View, CA). The laser was adjusted to 457 nm and emitted at 160–200 milliwatts. Fluorescence was measured through a Schott KV-250 long wave pass filter. Data were used to draw histograms of cell number *versus* fluorescence, which were analyzed at the Lawrence Livermore Laboratories with a program ("Peaks") that models fractions of cells in G_1 and G_2-M phases as Gaussian distributions and cells in S phase as a series of Gaussian distributions. A nonlinear least squares technique was used to model the data and to give the relative percentage of cells in each phase in the cell cycle (17).

RESULTS

Cell Growth

Continuous treatment of 9L cells with 1 mM DFMO or 1 mM DCHA produced a similar pattern of growth inhibition up to 56 h; after 56 h of treatment, however, growth inhibition of cells treated with DFMO continued and growth nearly plateaued at 120 h, while the growth rate of cells treated with DCHA increased to nearly control during the remainder of the experimental period (Chart 2). Cell growth was inhibited more effectively by treatment with the combination of 1 mM DCHA and 1 mM DFMO than by treatment with either agent alone; under these conditions cell growth ceased at 80 h of treatment.

Polyamine Levels

DCHA alone (1 mM) blocked the conversion of PU to SD, which caused PU levels to almost double by 2 h of treatment (Chart 3*A*); by 53 h PU levels were 350% of control levels and continued to increase until 80 h of treatment, at which time the level appeared to plateau at approximately 450%. SD levels decreased rapidly to approximately 50% of control by 5 h (Chart 3*B*), and levels were approximately 5% of control by 15 h after treatment was begun. SP levels were not significantly affected by treatment with DCHA (Chart 3*C*).

As found in earlier studies (18–20) treatment with 1 mM DFMO caused PU levels to decrease to less than 50% of control after

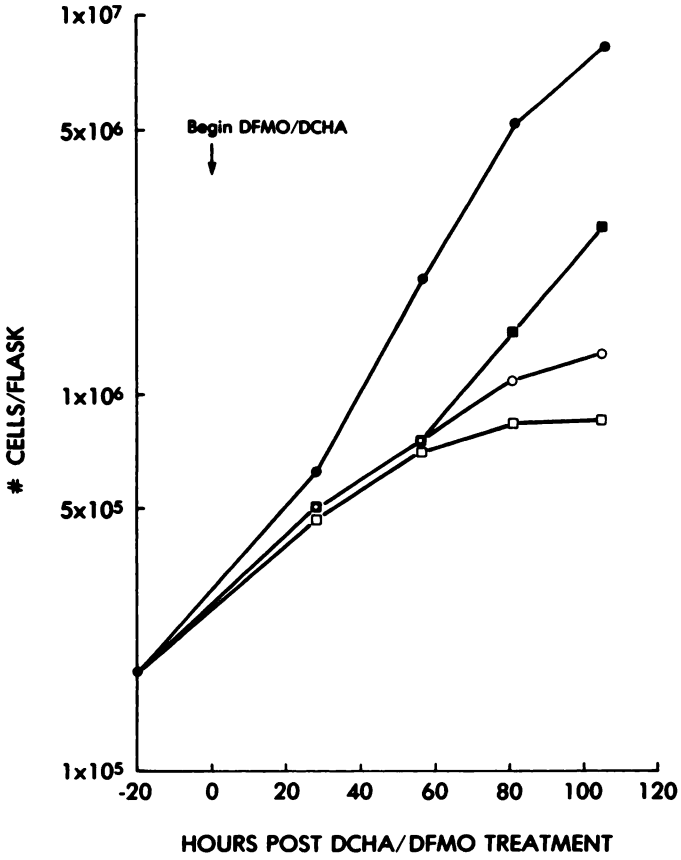

Chart 2. Effect of 1 mM DCHA and 1 mM DFMO on the growth of 9L cells. ●, control; ■, 1 mM DCHA; ○, 1 mM DFMO; □, 1 mM DCHA + 1 mM DFMO.

2 h of treatment and to nearly undetectable levels after 15 h of treatment (Chart 3*A*). Levels of SD were also very low after 15 h of treatment (Chart 3*B*), but the response time was somewhat delayed compared to both the decline of SD levels after treatment with DCHA and the decline of PU levels after treatment with DFMO. There was little change in SP levels after 48 h of treatment with DFMO, but by 80 h of treatment levels had fallen to 50% of control (Chart 3*C*).

Treatment with the combination of 1 mM DFMO and 1 mM DCHA lowered the cellular level of PU, but neither as rapidly nor as completely as treatment with DFMO alone. PU levels were approximately 50% of control after 5 h of treatment and essentially remained at that level for the remainder of the treatment period (Chart 3*A*). SD levels declined to approximately 25% of control by 5 h and reached nearly undetectable levels by 15 h of treatment (Chart 3*B*). SP was essentially the same as the control at 48 h and fell to approximately 50% of control by 80 h of treatment (Chart 3*C*), the same pattern found for treatment with DFMO alone.

Effect of Polyamine Depletion on Survival after Treatment with BCNU

Treatment with 20 μM BCNU killed slightly more than 1 log of both control and DCHA pretreated cells but killed approximately 2 logs of cells pretreated with DFMO and the DFMO/DCHA combination (Chart 4). Treatment with 35 μM BCNU killed approximately 3 logs of control and DCHA pretreated cells, almost

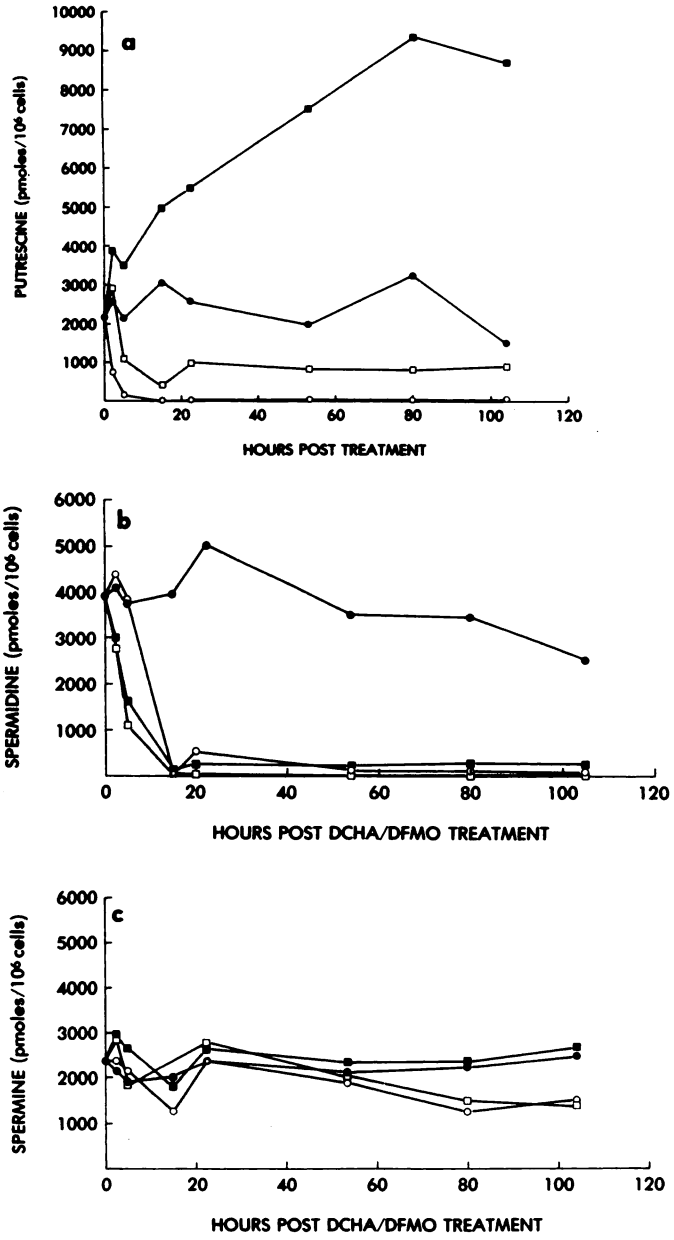

Chart 3. Effect of DCHA and DFMO on PU, SD, and SP levels in 9L cells. ●, control; ■, 1 mM DCHA; ○, 1 mM DFMO; □, 1 mM DCHA + 1 mM DFMO.

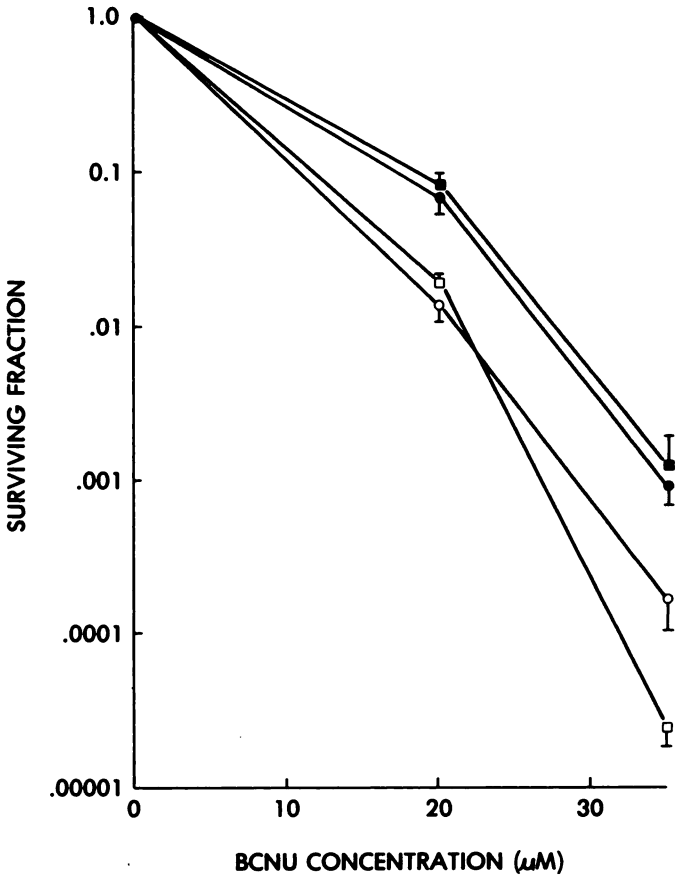

Chart 4. Effect of 1 mM DCHA and 1 mM DFMO pretreatment on the survival of 9L cells treated with BCNU. ●, control; ■, 1 mM DCHA, 48 h; ○, 1 mM DFMO, 48 h; □, 1 mM DCHA + 1 mM DFMO, 48 h. *Symbols*, means of 4 to 8 dishes; *bars*, SD.

Table 1

Effect of addition of PU on polyamines in 9L cells after treatment with DFMO[a]

	% ± SE of control value		
	PU	SD	SP
Control	100 ± 8	100 ± 15	100 ± 12
DFMO	ND[a]	3[b]	90 ± 10
DFMO + PU	420 ± 34	15[b]	77 ± 10
DFMO + DCHA + PU	481 ± 31	3[b]	83 ± 14

[a] ND, not detectable.
[b] The SE could not be determined because levels were at the limit of sensitivity of the assay.

4 logs of DFMO pretreated cells, and approximately 4.5 logs of cells pretreated with the DFMO/DCHA combination. Compared to controls the dose enhancement ratio at a surviving fraction of 0.001 was 1.3 for DFMO and 1.4 for the DFMO/DCHA combination. Pretreatment with DCHA caused no enhancement of BCNU cytotoxicity.

Putrescine Replenishment Experiments

Polyamine Levels. When 5 mM PU was added to the culture medium of cells pretreated with DFMO for 1 h, the intracellular PU level increased to over 400% of untreated controls (Table 1). SD levels, which were depleted to 3% of control by treatment with DFMO, were increased to 15% of control when exogenous PU was added to cultures for 1 h (Table 1). SP levels decreased slightly after PU was added to cultures.

If the metabolism of PU to SD was blocked by treatment with DCHA before PU was added to culture medium, PU levels (481% of untreated control) and SP levels (83% of untreated control) were essentially the same as levels in cells treated with PU alone 1 h after addition of PU. The SD levels, at 3% of untreated controls, were not changed (Table 1).

Cell Survival. Survival plots for PU replenished cells treated with BCNU are shown in Chart 5. The addition of 5 mM PU alone had no effect on BCNU cytotoxicity. As shown above (Chart 4) the cytotoxicity of BCNU was potentiated after treatment with DFMO. When PU levels were replenished in cells pretreated with DFMO, however, the potentiation was partially prevented. After treatment with 30 μM BCNU the 1.5-log increase in cell kill caused by pretreatment for 48 h with DFMO was decreased to 1 log by a 1-h treatment with PU and to 0.5 log by a 1-h treatment with

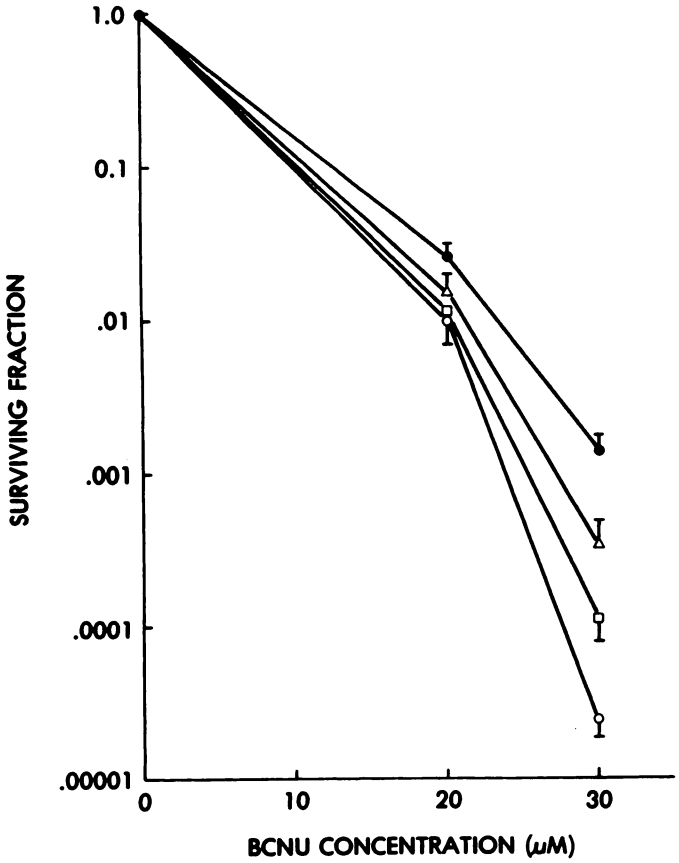

Chart 5. Effects of addition of exogenous PU on 9L cells treated with DFMO and BCNU. ●, control; ○, 1 mM DFMO, 48 h; □, 1 mM DFMO, 48 h, and 5 mM PU, 1 h; △, 1 mM DFMO, 48 h + 1 mM DCHA, 2 h + 5 mM PU, 1 h; *bars*, SD.

Table 2

Effect of treatment with 1 mM DCHA on cell cycle progression

Treatment time (h)	% of control value ± SE		
	G_1	S	G_2-M
0–15 ($N = 5$)[a]	101 ± 6	103 ± 13	90 ± 23
15–30 ($N = 11$)	83 ± 14	149 ± 16	47 ± 15
30–96 ($N = 9$)	99 ± 8	99 ± 15	93 ± 17

[a] Number of determinations are given in parentheses.

DCHA and PU. The dose enhancement ratios at a surviving fraction of 0.001 were 1.4 for DFMO, 1.3 for DFMO and PU, and 1.2 for DFMO, DCHA, and PU.

FCM

The results of several experiments in which treatment with 1 mM DCHA caused a slight but definite cell cycle block at the S-G_2 border between 15 to 30 h of treatment are summarized in Table 2. Formation of the block and release from it corresponded temporally to the depletion of SD and then with the accumulation of PU.

DISCUSSION

We have reported that the cytotoxicity of BCNU and other chloroethylnitrosoureas is potentiated by pretreatment with the polyamine biosynthesis inhibitors DFMO (11) and MGBG (2). MGBG blocks the metabolism of putrescine to spermidine by inhibiting *S*-adenosylmethionine decarboxylase (21). Because DCHA also inhibits the metabolism of PU to SD, albeit by blocking the action of spermidine synthase, it might be expected that DCHA would mimic the effects produced by pretreatment of 9L cells with MGBG. In these experiments, however, DCHA did not potentiate the cytotoxicity of BCNU. There are several possible reasons for this finding.

Because BCNU is more cytotoxic against 9L cells in G_1 and G_2 and less cytotoxic against cells in S phase (22), it is possible that a cell cycle block caused by DCHA could account for the lack of potentiation. FCM studies showed that treatment of 9L cells with DCHA caused only a slight, transient S-G_2 block that was released by 30 h after treatment was begun (Table 2). It is doubtful that this block would affect the cytotoxicity of BCNU given 48 h after the beginning of treatment with DCHA.

It is possible that the absence of potentiation was the result of a direct effect of DCHA on BCNU cytotoxicity. If a direct effect occurred the surviving fraction of cells treated simultaneously with DCHA and BCNU should be increased compared to cells treated with BCNU alone. This effect was not found in 9L cells treated simultaneously with DCHA and BCNU for 1 h (data not shown). In addition if DCHA directly affected BCNU cytotoxicity, the surviving fraction of cells treated simultaneously with DCHA and DFMO should be higher than the surviving fraction of cells treated with DFMO alone. No effect was found experimentally (Chart 4).

It is also possible that the effect of DCHA on polyamine pools could account for the lack of potentiation. By 48 h of treatment the intracellular levels of PU are approximately 400% of control (Chart 3). It is possible that, because of dramatically elevated concentrations, PU might substitute for SD and thereby prevent the potentiation of BCNU toxicity observed in cells pretreated with DFMO and MGBG. It is particularly important to consider the similarities and differences between MGBG and DCHA as inhibitors of polyamine biosynthesis (14, 21). Even though MGBG and DCHA both block conversion of PU to SD, different enzyme pathways are involved and the pattern of polyamine depletion caused by treatment with each agent is different in 9L cells. After 48 h of treatment with MGBG, 9L cells are partially depleted of SD and SP, and levels of PU are only slightly elevated (2). Thus MGBG potentiation of BCNU cytotoxicity in 9L cells might be caused by the depletion of PU to levels that are not sufficiently high to allow substitution of PU for the functions of the other polyamines. In addition SP is depleted by MGBG but not by DCHA.

The growth rate of 9L cells treated with DFMO alone and with the combination of DCHA and DFMO progressively declines during continuous treatment (Chart 2). The growth of 9L cells treated with DCHA alone, however, declines for the first 2 days of treatment but increases thereafter. The change occurs during the period in which PU levels are increasing (Chart 3*A*) and SD levels are fully depleted, suggesting that if 9L cells are depleted of SD and PU (DFMO treatment) growth is inhibited but that if PU levels are sufficiently high when SD levels are depleted (DCHA treatment) inhibition of growth can be reversed.

FCM data summarized in Table 2 suggest that PU alone also can reverse the initial perturbation in the cell cycle caused by DCHA. SD levels are fully depleted between 5 and 15 h of treatment with DCHA; this occurs when the slight but significant

block between S and G_2 phases appears. As PU levels approach 200–300% of control levels by 30 h of treatment, the cell cycle block is released. This suggests that in 9L cells 2- to 3-fold elevations in PU levels can overcome the cell cycle block caused by SD depletion.

Potentiation of BCNU cytotoxicity in 9L cells treated for 48 h with DFMO could be prevented partially either by adding exogenous PU alone (5 mM) to cell cultures or by treating cells with DCHA and PU after DFMO pretreatment (Chart 5). Intracellular levels of PU after a 1-h treatment with exogenous PU (after DFMO treatment) were comparable to levels caused by a 48-h treatment with DCHA (Chart 3; Table 1). Thus by mimicking the polyamine levels caused by 48 h of treatment with DCHA, the effect on BCNU cytotoxicity caused by treatment with DFMO can be prevented partially.

It is well known that PU can reverse or prevent the effects of DFMO treatment both on growth and on the cytotoxicity of BCNU (1, 5). It is generally assumed, however, that metabolism of PU to SD is necessary for the rescue to occur. The results reported here suggest that further metabolism of PU to SD may not be a necessary condition either for the reversal of growth inhibition or for prevention of the potentiation phenomena. In many cell systems treated with MGBG, supranormal levels of PU are produced with the simultaneous depletion of SD levels (19, 20). It has not been shown, however, that PU can reverse or prevent the effects of polyamine depletion, possibly because MGBG depletes both SD and SP levels (19, 20). Depletion of SP may be more detrimental to cells than is depletion of SD, or depletion of both simultaneously could be the critical event. In addition because MGBG is a SD analogue (23–25), MGBG may substitute directly for SD and produce additional toxicity. It is also possible that the cytotoxic effect of MGBG is not related to polyamine depletion (24).

We have proposed that alterations in the cytotoxicities of various DNA directed anticancer agents caused by polyamine depletion in 9L cells (1, 4, 11, 15) reflect changes in DNA structure caused by polyamine depletion (11). Our published results suggested that SD and perhaps SP were of more importance than PU for the control of DNA conformation. The results reported here suggest that, if intracellular levels are sufficiently high, PU can replace the function of the other polyamines, perhaps maintaining the conformational integrity of DNA. The fact that the conformation of synthetic DNA heteropolymers can be changed from the B to the Z form by treatment with polyamines, including PU, supports this idea (10). It is also of interest that the times needed to cause the heteropolymer B-Z transitions are well within the 1-h range during which PU treatment can reverse the effect of polyamine depletion of BCNU cytotoxicity (10).

Compounds other than polyamines are affected directly by inhibition of polyamine biosynthesis. Decarboxylated adenosylmethionine provides the aminopropyl group necessary for both SD and SP synthesis. It is possible that alteration in the levels of this compound caused by DCHA treatment could be a factor in explaining our results. This possibility is being investigated.

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