

Enhancement of the Rate of Spontaneous Mutation to 6-Thioguanine Resistance in Mammalian Cells by Polyamine Depletion¹

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ABSTRACT

Several lines of evidence have suggested, but not proved, that polyamines are associated with DNA in intact cells. In an attempt to investigate the roles of polyamines in gene-associated functions, we examined the effects of polyamine depletion on the spontaneous mutation rate in a rat basophilic leukemia cell line. The frequency of 6-thioguanine-resistant mutant cells increased by approximately 9-fold as a result of the treatment with α -difluoromethylornithine, a potent inhibitor of ornithine decarboxylase (EC 4.1.1.17). This increase was prevented by supplementing the cultures with putrescine, suggesting that polyamine depletion, but not the direct mutagenic action of the enzyme inhibitor, is responsible for the mutant-increasing effect. These results suggest that polyamines may participate in the conservation of genetic information at either the chromosome or gene level.

INTRODUCTION

Several lines of evidence have suggested that the physiological roles of the polyamines (putrescine, spermidine, and spermine) are closely associated with DNA. Thus: (a) a dramatic elevation of the enzymes for polyamine biosynthesis and subsequent rise in the level of each polyamine either precede or coincide with almost all the DNA replication events (for reviews, see Refs. 2, 15, 30, and 34); (b) in test tubes, polyamine molecules bind tightly to purified DNA (11) and protect the latter compound from denaturation and shearing (35); (c) such DNA-polyamine association is actually playing an important role in the packaging of DNA in *Escherichia coli* bacteriophage T4 (1) and herpes virus (9); and (d) in *in vitro* reactions, polyamines affect DNA synthesis (10), DNA-dependent RNA synthesis (5), DNA methylation (6), and DNA supercoiling (24).

It has been possible to reduce the polyamine contents in dividing mammalian cells by either the treatment with enzyme inhibitors (14, 17, 23, 27, 28) or by mutational enzyme deficiency (33). The growth of these polyamine-depleted cells slowed down (14, 17, 23, 27). When the deficiency was severe, the cells eventually stopped growing and died (33). Such polyamine-deficient states rendered the cells susceptible to the DNA-damaging effects of 1,3-bis(2-chloroethyl)-1-nitrosourea (12, 36) as evidenced by the decrease in the percentage of the cells that survived (12) or the increase in the frequency of the sister chromatid exchange (36) after the mutagen treatment. The polyamine depletion also increased the sensitivity of mouse or rat

tumor cells to Adriamycin or videsine (3).

These previous studies prompted us to investigate whether the data programmed in DNA are protected by the polyamines not only when exogenous mutagens are added, but under regular conditions as well. In order to increase the sensitivity, we, unlike previous investigators, attempted to demonstrate the changes in DNA from the aspect of the phenotypic expression.

In the present investigations, we depleted cultured rat cells of the polyamines, putrescine and spermidine (but not spermine), by treatment with DFMO.³ Such polyamine-depleted cells allowed us to examine the effects of the polyamine depletion on the frequency of the mutation with respect to the HGPRT locus (4).

MATERIALS AND METHODS

Cell Culture. RBL cells (8) were obtained from Dr. S. Ida at Tohoku University and maintained in Eagle's minimal essential medium supplemented with 100 units of penicillin per ml, 100 μ g of streptomycin per ml, and 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) at 37° in a humidified atmosphere containing 95% air and 5% CO₂.

Treatment of Cells with DFMO. RBL cells in log-phase growth were detached from the culture dish with a rubber policeman, and a 5-ml cell suspension containing 2×10^5 cells was placed in each culture dish (Falcon No. 3003). Five ml of medium supplemented with DFMO alone, DFMO plus putrescine, or no drugs (control) were then added to the dishes. After a 48-hr culture under the conditions described above, the cells were processed for either the polyamine determination or the enumeration of thioguanine-resistant cells as described below.

Polyamine Determination. After the drug treatment, the cells were washed twice with PBS and then detached from the dish with rubber policeman. The cells in suspension thus prepared were washed again with PBS, and part of the cells was used for the counting of the viable cell number. The rest of the cells were centrifuged, and cold 0.4 N perchloric acid was added to the pellets. Then, the amines in the supernatants were dansylated under the conditions described by S. Osterberg *et al.* (26), extracted into toluene, and applied to silica gel thin-layer plates (Merck No. 5553; E. Merck, Darmstadt, West Germany). After development in benzene/triethylamine (5/1, v/v) in the first direction and in distilled water in the second direction, the fluorescent spots corresponding to dansylated putrescine, spermidine, and spermine were cut out and extracted into dioxane, and the fluorescent intensity was determined as described by Seiler (32).

Enumeration of Thioguanine-resistant Cells. After 7 days of culture in the regular medium, 2×10^5 cells were placed in a Falcon dish in 10 ml of medium containing 5 μ M thioguanine. After 7 additional days, the dish was washed with PBS and stained with Giemsa solution. The colonies were enumerated under a microscope as described previously (25). The effect of MNNG treatment on the number of thioguanine-

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³ The abbreviations used are: DFMO, α -difluoromethylornithine; HGPRT, hypoxanthine guanine phosphoribosyltransferase; RBL, rat basophilic leukemia; PBS, phosphate-buffered saline (0.15 M NaCl/0.01 M phosphate buffer, pH 7.4); MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.

resistant cells was tested as described previously (25), and, in this case, the culture in the selecting medium was performed in several dishes containing various cell numbers.

Materials. DFMO was a generous gift from Dr. D. J. Wilkins at the Centre de Recherche Merrell International (Strasbourg, France). Other chemicals were of the finest quality commercially available.

RESULTS AND DISCUSSION

Increase in Mutant Frequency after Treatment with MNNG. The rat basophilic cells were treated with the mutagen MNNG for 12 hr, and, after 7 days, the frequency of 6-thioguanine-resistant cells among the treated cells was enumerated as described in "Materials and Methods." Without mutagen treatment, the rat cell population maintained in our laboratory contained thioguanine-resistant cells at frequencies of around one per 10^5 cells. This frequency increased to 21 to 55 per 10^5 cells after the cells were treated with MNNG (Chart 1). This dose-dependent curve is generally in agreement with those in previous reports (25).

Polyamine Depletion Induced by DFMO. The rat cells were cultured for 2 days in medium containing 1.25 mM DFMO with or without $10 \mu\text{M}$ putrescine. The amounts of putrescine, spermidine, and spermine in washed cells were determined as described in "Materials and Methods." Table 1 shows that DFMO profoundly reduced the intracellular putrescine and spermidine, but not spermine. In the cells from the cultures containing both DFMO and putrescine, the polyamine depletion was partially prevented (Table 1). The mode of polyamine depletion induced by DFMO, *i.e.*, the severe decrease in putrescine and spermidine,

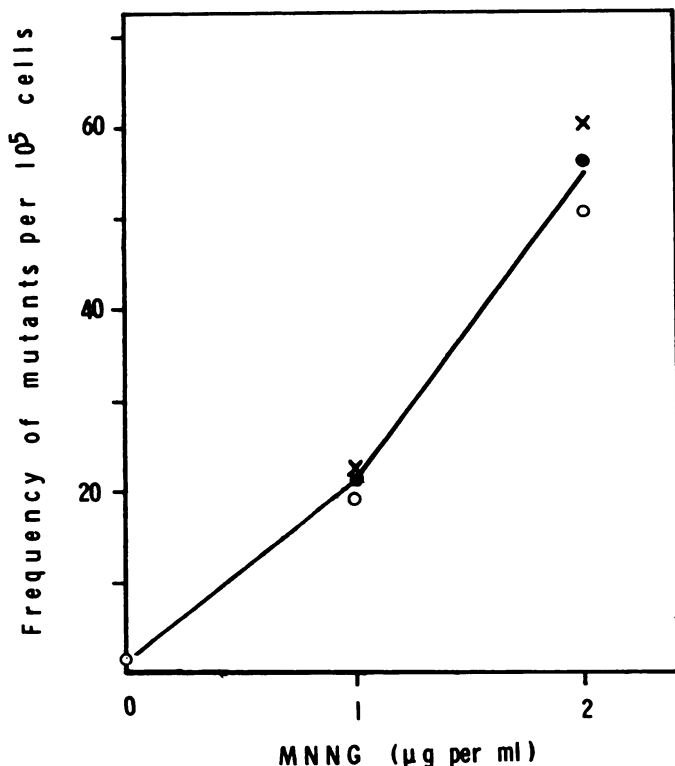


Chart 1. RBL cells were treated with MNNG at concentrations of 0, 1, and 2 $\mu\text{g/ml}$ for 12 hr, and thioguanine-resistant cells were enumerated as described in "Materials and Methods." Cells [10^5 (O), 2×10^5 (●), or 4×10^4 (x)] were placed in each culture dish in the thioguanine medium. Points, mean from duplicate cultures.

Table 1

Effect of DFMO on polyamine levels

RBL cells were cultured with 1.25 mM DFMO in the presence or absence of $10 \mu\text{M}$ putrescine for 48 hr, and intracellular polyamine levels were determined as outlined in "Materials and Methods."

	Putrescine	Spermidine	Spermine
Control	4.8 ± 0.3^a	26.6 ± 2.7	7.7 ± 1.2
DFMO	1.1 ± 0.2	2.8 ± 0.4	7.8 ± 0.6
DFMO + putrescine	3.1 ± 0.5	16.4 ± 2.1	6.9 ± 0.5

^a Mean \pm S.D. (nmol/ 10^7 cells) from 3 replicate cultures.

but not spermine, agrees with the reports by other researchers (31, 37). After the 2-day culture of the cells with the drugs, the cells were washed and then maintained in a drug-free medium. Reflecting the deficiency of the polyamines, the proliferation of the cells treated with DFMO alone was inhibited. Although the growth inhibition was not evident on the third day, the doubling time calculated between the third and fifth days was approximately 2.2 times as long for DFMO-treated cells as for the control cells. On the 10th day, however, the growth rate of the DFMO-treated cells was comparable to the control cultures. The growth of the cells treated with DFMO and putrescine was almost as good as control cells, but a slight prolongation of the doubling time (1.2 times as long as control) was observed.

Effects of Polyamine Depletion on Mutation. From the ninth day, the cells were propagated in the thioguanine-containing medium for enumerating the HGPRT-negative cells. Table 2 summarizes the results from 4 separate experiments. In each of the 4 experiments, the cultures treated with DFMO contained significantly higher numbers of thioguanine-resistant clones than control cultures (Table 2). The average number of the clones, however, varied between the experiments. Combined data from 4 experiments indicated that the average numbers of mutant clones per plate for the DFMO-treated and control cell populations were 18.3 ± 7.9 (S.D.) and 1.9 ± 1.4 , respectively. The cloning efficiency of this cell line before DFMO treatment and 7 days after removal of DFMO was 1.0. Taking this cloning efficiency as well as the number of cells applied to each dish into account, the cell populations in control and DFMO-treated cultures are calculated to contain 1.0 and 9.1, respectively, thioguanine-resistant cells per 10^5 cells (averages of the means from 4 experiments). Calculation from the same data indicates that new mutation to thioguanine resistance developed in 8.1 of 10^5 cells by the treatment with DFMO. Since both the growth rate and the degree of polyamine depletion mildly fluctuated during the treatment with DFMO, neither the mutation rate per generation nor the precise relation between the degree of polyamine depletion and the mutant frequency was determined. These data clearly indicated that significant numbers of new mutants appeared in the cell populations treated with DFMO, but not in control cultures. The possibility that this effect is not associated with polyamine depletion, but is due to the direct mutagenic action of DFMO, is unlikely because the supplementation of the DFMO-containing cultures with as little as $10 \mu\text{M}$ putrescine completely prevented the mutant-increasing effect of DFMO (Table 2).

Although the thioguanine-resistant clones of this exact cell line were not characterized, previous studies on various other cell lines have clearly shown that more than 98% of thioguanine-resistant clones, unlike 8-azaguanine-resistant clones, lack a purine-salvage enzyme, HGPRT (25). The following evidence strongly suggests that the occurrence of the thioguanine-resist-

Table 2
Number of 6-thioguanine-resistant clones per dish

After 2-day culture with 1.25 mM DFMO in the presence or absence of 10 μ M putrescine, the cells were propagated for 7 days in drug-free medium, and then thioguanine-resistant cells were enumerated as described in "Materials and Methods." In Experiments 1 to 3, only one thioguanine-containing plate was prepared for each culture, while the enumeration of the thioguanine-resistant clones was performed in 5 dishes per culture in Experiment 4.

Experiment	Control			DFMO			DFMO + putrescine		
	No. of thioguanine-resistant clones in a plate initially containing 2×10^6 cells at start of culture	Mean of nos. of clones in 5 dishes from each culture	Av. no. of clones/dish for each experiment	No. of thioguanine-resistant clones in a plate initially containing 2×10^6 cells at start of culture	Mean of nos. of clones in 5 dishes from each culture	Av. no. of clones/dish for each experiment	No. of thioguanine-resistant clones in a plate initially containing 2×10^6 cells at start of culture	Mean of nos. of clones in 5 dishes from each culture	Av. no. of clones/dish for each experiment
1	0, 0, 0		0	11, 10, 7		9.3	0, 0, 3		1.0
2	0, 12, 0		4.0	35, 40, 11		28.7	0, 0, 1		0.3
3	0, 1, 5		2.0	29, 18, 22		23.0	0, 6, 8		4.7
4			1.7			12.1			
Dish 1	0, 1, 0	0.3		12, 8, 13	11.0				
Dish 2	5, 0, 3	2.7		5, 13, 11	9.7				
Dish 3	3, 0, 0	1.0		4, 5, 7	5.3				
Dish 4	0, 0, 0	0.0		23, 12, 15	16.7				
Dish 5	6, 7, 1	4.7		16, 20, 18	18.0				
Av. nos/dish ^a		1.9 ± 1.4^b			18.3 ± 7.9^b			2.0 ± 1.9	
Frequency ^c		1.0×10^{-5}			9.1×10^{-5}			1.0×10^{-5}	

^a Average number of thioguanine-resistant clones calculated from the data from separate experiments.

^b $p < 0.05$ by Wilcoxon's nonpaired test.

^c Frequency of thioguanine-resistant mutants.

ant clones from this exact cell line, just as in other cell lines (25), involves a genetic alteration and therefore is considered to be a mutational event. Thus, MNNG, a DNA-alkylating agent, dose-dependently increased the frequency of the thioguanine-resistant clones in the RBL cell population (Chart 1). Moreover, the thioguanine-resistant characteristic of each selected clone persisted even after 14 cell doublings (data not presented).

The precise mechanisms by which polyamine depletion increases the mutation frequency remain to be elucidated. Although the association between the polyamines and DNA in test tubes has been extensively studied (2, 15, 30, 34), current knowledge does not seem to be sufficient to explain the phenomena in viable cells reported here. Considering previous data, 2 possibilities have to be kept in mind. (a) Polyamine depletion may change the structure of DNA or chromosomes. As stated earlier, polyamine depletion rendered mammalian cells sensitive to the DNA-damaging effects of some chemical compounds (12, 36). A recent report has stated that chromosomal aberration was induced by polyamine depletion without exogenous chemicals (18). (b) Alternatively, the accuracy of DNA replication may be impaired by polyamine depletion. If the association between the polyamines and DNA as shown in *in vitro* experiments also occurs in viable cells, it is likely that such association affects the fidelity of DNA replication. Other DNA-associated compounds, such as the product of bacteriophage T4, gene 32, and metal cations, have been reported to affect the frequency of replication errors (7, 19, 21, 39).

Indeed, mutation has been suggested as a major factor in carcinogenesis, aging, and evolution (20). Recent investigations have demonstrated that the imbalance in the physiological precursor pools for the DNA biosynthesis (deoxyribonucleoside triphosphate) enhanced the rate of spontaneous mutation (16, 29, 37, 38). The data presented here suggest that the derangement in the levels of the physiological cations, polyamines, may

also increase the rate of spontaneous mutation. Changes in the levels of these physiological polycations may affect the rate of carcinogenesis or the mode of aging or evolution. Of course, the data presented in the present report do not exclude other roles of the polyamines, such as those related to RNA and protein synthesis (13, 22, 23) or membrane functions.

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