MUTAGENESIS IN CULTURED HUMAN DIPLOID CELLS II. CHEMICAL INDUCTION OF 8-AZAGUANINE- RESISTANT MUTATIONS¹⁾

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The previous paper (Kuroda 1974) showed that human diploid cells derived from normal embryonic lung had the highest plating efficiency in Ham's medium F12 supplemented with 10 % calf serum and the D_0 values of some mutagens and a selective agent for cell survival were reported.

It has been found that the following factors influence the mutation of Chinese hamster cells by chemicals and X-rays: the inoculum size, the period between mutagen quenching and addition of a selective agent (mutation expression time), and the concentration of the selective agent (Chu and Malling 1968; Shapiro *et al.* 1972; Duncan and Brookes 1973).

In the present work human diploid cells were treated with ethyl methanesulfonate (EMS) and then cultured in selection medium containing 8-azaguanine (8AG), and the effects of the inoculum size and the mutation expression time on the frequency of 8-azaguanine-resistant mutations were examined.

MATERIALS AND METHODS

Cell cultures: The origin, culture procedures and properties of the human diploid cells used were described in the previous paper (Kuroda 1974). The cells were maintained and plated in Ham's medium F12 (1965) with 10 % calf serum. Human diploid cell strains die after a limited number of population doublings, of the order of 50, *in vitro* (Hayflick and Moorhead 1961). Therefore, cells at the fifteenth to seventeenth generation were used in the present work.

Induction of 8-AG-resistant mutations: Cells were dissociated from monolayers by trypsinization and inoculated into 3.6 ml of normal medium in 60-mm plastic petri dishes. After incubation for 2 hours at 37°C, 0.4 ml of medium containing 10^{-1} M EMS was added and incubation was continued for 2 hours. This treatment with EMS at a final concentration of 10^{-2} M resulted in 46 % cell survival (Kuroda 1974). After this treat-

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ment the cells were rinsed three times with Tyrode's solution and incubated in normal medium for an appropriate period (mutation expression time). Then the medium was replaced by selection medium containing 10 μ g/ml or 30 μ g/ml of 8AG.

After a total cultivation period of 14 days the cell colonies in each dish were fixed with methyl alcohol, and stained with May-Grünwald-Giemsa solution. The number of colonies containing more than 50 cells was counted under a binocular microscope. In cultures without EMS treatment the number of colonies produced was frequently more than 2,000, causing a difficulty in calculating the colony. In this case the number of colonies containing more than 20 cells were scored after 7 days of cultivation.

Determination of the induced mutation frequency: The number of 8AG-resistant mutant colonies induced by EMS was corrected for the decrease in cell survival (per cent survival) due to the cytotoxic action of EMS. The number of induced mutants was calculated by subtracting the number of colonies in untreated control cultures from that in EMS-treated cultures. The number of colonies in control cultures, included colonies of mutants present among the cells plated initially, as well as those arising during the experiment. The induced mutation frequency was expressed as the number of induced mutants per 10^5 survivors.

Examination of cell growth in colonies: 10^4 cells were inoculated, treated with 10^{-2} M EMS for 2 hours, washed with Tyrode's solution, and incubated in normal medium at 37°C. The average number of cells per colony was determined by direct microscopic observation on 150 colonies in replicate cultures fixed 2, 24, 48, 72 and 96 hours after removal of the mutagen. The number of cells per colony was also determined in the same way in untreated control cultures, after rinsing with Tyrode's solution and changing the medium, as in test cultures.

RESULTS

Effect of inoculum size on mutation frequency

Two experiments were carried out for examining the effect of inoculum size on the frequency of 8AG-resistant mutations induced by EMS using two levels of 8AG for selection. In the first experiment 5×10^4 , 10^5 , 2.5×10^5 and 5×10^5 cells were inoculated into petri dishes and treated with 10^{-2} M EMS for 2 hours. Then the cells were incubated for 48 hours in normal medium (mutation expression time), and selected in medium containing 10 μ g/ml of 8AG. The results are shown in Table 1. In this experiment 10^{-2} M EMS was found to have a cytotoxic action, reducing cell survival to 40.2 to 75.4 % depending on the inoculum size. The mutation frequency was highest on inoculation of 2.5×10^5 cells and second highest on inoculation of 10^5 cells. These high mutation frequencies obtained on inoculations of 2.5×10^5 and 10^5 cells, however, were not significant statistically. On inoculations of 5×10^4 and 5×10^5 cells, 8AG had a synergistic lethal effect when combined with EMS, producing less corrected numbers of colonies than those without EMS treatment.

In the second experiment 10^5 , 2.5×10^5 , 5×10^5 and 10^6 cells were inoculated into

Table 1. Relation of inoculum size to 8AG-resistant mutation frequency in cultured human diploid cells selected with 10 μ g/ml of 8AG*

Number of cells per plate	EMS treatment**	8AG selection***	Number of colonies formed	Average number of colonies	Per cent survival	Corrected number of colonies	Number of induced mutants	Induced mutation frequency per 10 ⁵ survivors
5 $\times$ 10 ⁴	—	—	3184, 2052, 2844	2693.3	100.0			
		+	36, 23, 14	24.3		24.3		
	+	—	1808	1808.0	67.1		—7.9	—15.8
10 ⁵	—	+	11, 11	11.0		16.4		
		—	3740, 3716	3728.0	100.0			
	+	—	26, 29, 17	24.0		24.0		
2.5 $\times$ 10 ⁵	—	—	2312, 1868, 1816	1998.7	53.6		10.9	10.9
		+	17, 23, 16	18.7		34.9		
	+	—	4600, 4624, 3784	4336.0	100.0			
5 $\times$ 10 ⁵	—	+	46, 30, 27	34.3		34.3		
		—	2336, 1328, 1568	1744.0	40.2		43.6	17.4
	+	—	24, 16, 54	31.3		77.9		
5 $\times$ 10 ⁵	—	—	5104, 4800, 5128	5010.7	100.0			
		+	75, 79, 57	70.3		70.3		
	+	—	3728, 4112, 3488	3776.0	75.4		—18.6	—3.7
		+	39, 42, 36	39.0		51.7		

* Cells at the fifteenth generation were used.

** Treated with 10⁻² M EMS for 2 hours.*** Selected with 10 μ g/ml of 8AG after an expression time of 48 hours.

petri dishes. Treatment with EMS and the duration of mutation expression time were as in the first experiment, but 8AG-resistant cells were selected in medium containing 30 μ g/ml of 8AG. The results shown in Table 2 indicate that the highest frequency of cells resistant to 30 μ g/ml of 8AG was obtained on inoculation of 10⁵ cells. This mutation frequency induced by EMS was significantly higher than in cultures without EMS treatment ($F=28.59$, $\alpha=0.01$). The mutation frequency was lower with a larger inoculum.

Effect of mutation expression time on mutation frequency

Next the effect of the mutation expression time on the frequency of 8AG-resistant mutations in human diploid cells was examined. 10⁵ cells were plated and treated with 10⁻² M EMS for 2 hours. After rinsing with Tyrode's solution, the cells were incubated in normal medium for 0, 24, 48, 72 and 96 hours. Then 8AG was added at a final concentration of 30 μ g/ml.

After a total incubation period of 14 days, the number of cell colonies formed in each dish was recorded and the induced mutation frequency was calculated. Table 3 shows the results. In control, unselected cultures 10⁻² M EMS had a relatively severe cytotoxic effect resulting in 13.4 % cell survival.

The induced mutation frequency was higher in cultures with a mutation expres-

Table 2. Relation of inoculum size to 8AG-resistant mutation frequency in cultured human diploid cells selected with 30 μ g/ml of 8AG*

Number of cells per plate	EMS treatment**	8AG selection***	Number of colonies formed	Average number of colonies	Per cent survival	Corrected number of colonies	Number of induced mutants	Induced mutation frequency per 10 ⁵ survivors
10 ⁵	—	—	2096, 2416, 2344	2285.3	100.0			
		+	10, 9, 9	9.3		9.3		
	+	—	1488, 1136, 1608	1410.7	61.7		8.0	8.0
2.5×10 ⁵	—	+	12, 11, 9	10.7		17.3		
		—	2920, 2928	2924.0	100.0			
	+	—	27, 16	21.5		21.5		
5×10 ⁵	—	+	3024, 2824	2924.0	100.0		8.5	3.4
		+	30	30.0		30.0		
	+	—	3496	3496.0	100.0			
10 ⁶	—	+	37	37.0		37.0		
		—	3392	3392.0	97.0		4.2	0.8
	+	—	44, 36	40.0		41.2		
10 ⁶	—	+	5582	5582.0	100.0			
		+	30	30.0		30.0		
	+	—	5416	5416.0	97.0		29.8	3.0
		+	58	58.0		59.8		

* Cells at the seventeenth generation were used.

** Treated with 10⁻² M EMS for 2 hours.*** Selected with 30 μ g/ml of 8AG after an expression time of 48 hours.

Table 3. Relation of mutation expression time to 8AG-resistant mutation frequency in cultured human diploid cells*

Expression time (hr)	EMS treatment**	8AG selection***	Number of colonies formed	Average number of colonies	Per cent survival	Corrected number of colonies	Number of induced mutants	Induced mutation frequency per 10 ⁵ survivors
Control	—	—	2936, 3560, 2976	3157.3	100.0			
	+	—	560, 288	424.0	13.4			
0	—	+	0, 0, 0	0		0	—	—
	+	+	0, 0, 0	0		0		
24	—	+	1, 2, 1	1.3		1.3		
	+	+	3, 0, 2	1.7		12.7	11.4	25.4
48	—	+	4, 5	4.5		4.5		
	+	+	4, 4	4.0		29.9	25.4	25.4
72	—	+	11, 6, 6	7.7		7.7		
	+	+	4, 4, 5	4.3		32.1	24.4	24.4
96	—	+	17, 10, 9	12.0		12.0		
	+	+	8, 9, 13	10.0		74.6	62.6	62.6

* 10⁵ cells at the seventeenth generation were plated per dish.** Treated with 10⁻² M EMS for 2 hours.*** Selected with 30 μ g/ml of 8AG after the expression times indicated.

sion time of 48 hours than in cultures with slightly longer or shorter mutation expression times, but was highest with a mutation expression time of 96 hours.

Proliferation of cells treated with EMS

To investigate the relationship between the mutation expression time and cell growth, the number of cells in colonies was examined. Fig. 1 represents the number of cells per colony after various periods of cultivation in normal control medium. After cultivation for 2 hours only single cells were observed. After 24 hours about one third of the cells had divided once or twice and the average number of cells per colony was 1.49, or $2^{0.58}$. After 48 hours more cells had divided, although two fifths of the cells remained single, and the average number of cells per colony was 2.04, or

Fig. 1. Cell numbers in colonies of human diploid cells after different periods of growth in normal medium at 37°C.

Fig. 2. Cell numbers in colonies of human diploid cells treated with 10^{-2} M EMS for 2 hours and then cultured in normal medium for various periods.

2^{1.03}. After 72 hours most cells had divided at least once, giving an average number of cells per colony of 6.29, or 2^{2.65}. After 96 hours colonies contained from 1 to 54 cells and the average number of cells per colony was 21.64, or 2^{4.44}.

From these results, the average time for cell doubling of these human diploid cells was estimated as approximately 40 to 50 hours in the initial 48 hours of cultivation and after this period as about 14 to 15 hours.

Cells treated with 10⁻² M EMS for 2 hours had a somewhat different pattern of cell growth in normal medium, as shown in Fig. 2. A similar pattern of cell growth to that in control cultures was observed 24 hours after removal of mutagen and the average number of cells per colony was 1.45, or 2^{0.54}. The number of cells in colonies remained unchanged for another 24 hours and the average number of cells per colony after incubation for 48 hours was 1.44, or 2^{0.53}. Then the cell number increased, but increase was slower than that of untreated cells in control cultures. About four fifths of the cells had divided at least once 72 hours after removal of mutagen and the average number of cells per colony was 3.97, or 2^{1.99}. Then the cells continued to divide and after 96 hours the average number of cells was 4.92, or 2^{2.30}.

These results show that EMS-treated cells divided at a similar rate to untreated cells in control cultures for the initial 24 hours after removal of mutagen, but that after 72 hours the time of population doubling was more than 77 hours. Thus the highest mutation frequency was obtained when selection with 8AG was carried out after about half the EMS-treated cells had divided.

DISCUSSION

It has been found that an inoculum size of 2.5×10⁵ Chinese hamster cells per 100-mm dish produces the highest frequency of EMS-induced 8AG-resistant mutations (Chu and Malling 1968). In a reconstruction experiment to assess the efficiency of recovery of 8AG-resistant mutant cells from a culture which also contained normal cells, the maximum recovery was achieved with an initial inoculum of 2×10⁴ normal cells per 60-mm culture dish (Albertini and DeMars 1973). Similar findings have been reported for mouse and human diploid cells, and good recoveries of mutant cells were obtained at a density of about 190 wild-type cells per cm² (10⁴ cells per 90-mm dish) in reconstructed mixed cultures (van Zeeland *et al.* 1972). In the present experiments with human diploid cells the highest frequency of EMS-induced mutations was obtained on inoculation of 2.5×10⁵ cells per 60-mm dish and selection with 8AG at a level of 10 µg/ml. On selection with 30 µg/ml of 8AG the highest mutation frequency was observed on inoculation of 10⁵ cells per dish, and a larger inoculum reduced the mutation frequency. This reduction is probably due to "metabolic cooperation", which can be defined as the process by which mutant cells incorporate some relevant enzyme or its precursor on cocultivation with normal cells (van Zeeland *et al.* 1972). This was demonstrated in studies on single allele expression of human cells heterozygous for hypoxanthine-guanine-phosphoribosyl-transferase(HGPRT)-deficiency (Salzman *et al.* 1968; Fujimoto and Seegmiller 1970).

It is known that 8AG-resistant mutations, which result in decrease or complete absence of HGPRT activity, are recessive. This has been confirmed by analysis of the pedigrees of patients with the Lesch-Nyhan syndrome (Nyhan 1968) and by many experiments on hybridization of somatic cells (Littlefield 1964; Marin 1969; Chu *et al.* 1969; Klebe *et al.* 1970). The human gene controlling HGPRT activity is sex-linked. The human diploid cells employed in the present experiments had the normal female karyotype with two X-chromosomes (Kuroda 1974). The finding that 8AG-resistant mutants were induced in relatively high frequency by EMS suggests the following two possible explanations for the expression of a recessive mutant phenotype. (a) These female human cells have two active X-chromosome, one carrying the allele HGPRT⁻, and the other the HGPRT⁺ gene and mutations from HGPRT⁺ to HGPRT⁻ are induced on the latter chromosome. (b) In female human cells only one X-chromosome is active (Salzman *et al.* 1968; Cohen and Rattazzi 1971) and the gene mutation from HGPRT⁺ to HGPRT⁻ arising on the active X-chromosome can be expressed phenotypically, since the other X-chromosome carrying the dominant allele is inactive.

In the present experiment cells surviving in 8AG-containing medium may involve cells carrying the HGPRT⁺ gene, since Ham's medium F12 contained hypoxanthine, which may be useful for 8AG-sensitive cells to survive. This probability, however, is not likely, because the sensitivity of human diploid cells to 8AG in Ham's medium F12 corresponded closely to those reported for other human diploid cells in hypoxanthine-deficient medium (Kuroda, 1974).

It was reported that the maximum number of 8AG-resistant mutations of Chinese hamster cells was observed when the selective agent was added 42 hours after removal of mutagen—a time interval permitting approximately three cell divisions (Chu and Malling 1968). In the induction of 8AG-resistant mutations by carcinogenic hydrocarbons the mutation frequency increased arithmetically with the number of cell divisions occurring before addition of the selective agent, and reached a maximum after 3 to 4 divisions (Duncan and Brooks 1973).

In another line of Chinese hamster cells the highest yield of mutations to 6-mercaptopurine (6MP) resistance induced by chemicals and X-rays was observed when cells were placed in selective medium during several generations after treatment with mutagen. On the other hand, the maximum yields of mutations to 6MP-resistance induced by UV-light and to glucose-independence induced by *N*-nitrosomethyl-urea were recorded when cells were transferred to selective media immediately after treatment (Shapiro *et al.* 1972).

In the present experiments the highest yield of mutations to EMS-induced 8AG-resistance was observed when cells were selected in medium containing 8AG 48 hours after EMS treatment. This time interval corresponds to a period in which about half the cells divided once.

One possible explanation for the delayed expression of the 8AG-resistant mutant phenotype is that after mutation cells still contain considerable quantities of residual HGPRT enzyme or its messenger RNA in their cytoplasm. Another possibility is based on the mode of action of EMS in producing gene mutation. Namely, alkylating agents such as EMS react with nucleotide bases of DNA, producing a base-pair transi-

tion (Freese 1963). Thus it seems likely that DNA replication is necessary for phenotypic expression of gene mutation in single strand DNA.

The pattern of cell proliferation in colonies of human diploid cells is very interesting. Unlike in cultures of most established cell lines single cells plated in petri dishes did not divide equally. If cells divide synchronously, the number of cells in colonies increases exponentially, as depicted in Fig. 3a. In this case the number of cells will be 2^n after n cell divisions. On the other hand, if after each cell division only one daughter cell divides, the number of cells will be $n+1$ after n cell divisions, as represented in Fig. 3b.

In the present experiments human diploid cells in control cultures divided asynchronously and after cultivation for 96 hours the number of cells in colonies varied widely. This suggests that some cells in the population divided synchronously and grew exponentially, whereas others divided several times in the normal way, and then only one daughter cell continued to divide after each cell division as described above. In the latter cell fractions, when cell colonies were dissociated into single cells and plated on dishes, many cells were abortive and did not divide in further cultivation.

In cultures of EMS-treated cells the pattern of cell growth was essentially the same as that of untreated cells in control cultures, although their division rate was lower. In the mutation expression time which gave the highest induced mutation frequency the average number of cell divisions was 0.53 and some cells divided more than twice. If the fraction of many abortive cells in population was taken into consideration, this number of cell divisions giving the highest induced mutation frequency may be underestimated. It will be interesting to determine whether EMS induces mutations to 8AG-resistance preferentially in human diploid cells which divide exponentially.

Fig. 3. Patterns of cell proliferation. (a) Exponential cell growth. The number of cells is 2^n after n cell divisions. (b) After each cell division, only one daughter continues to divide. The number of cells is $n+1$ after n cell divisions.

SUMMARY

Human diploid cells were treated with ethyl methanesulfonate (EMS) and cultured in selection medium containing 8-azaguanine (8AG) to induce mutation to 8AG-resistance. The effects of factors such as the inoculum size and the mutation expression time on the induced mutation frequency of cells were examined. When cells were treated with 10^{-2} M EMS for 2 hours and selected with $10\text{ }\mu\text{g/ml}$ of 8AG, the induced mutation frequency was highest on inoculation of 2.5×10^5 cells, although this frequency was not significant statistically. When EMS-treated cells were selected with $30\text{ }\mu\text{g/ml}$ of 8AG, the highest induced mutation frequency was obtained on inoculation of 10^5 cells. The mutation frequency was higher when 10^5 cells treated with EMS were cultured in normal medium for 48 hours (mutation expression time) before selection with 8AG, than in cultures with longer or shorter mutation expression times. This mutation expression time of 48 hours corresponds to the time during which about half the cell population treated with EMS divides once. Microscopic examination on the numbers of cells in colonies indicated that human diploid cells treated with or without EMS do not all grow exponentially but that in some cases after each cell division only one daughter cell continued to divide.

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