Chromosomal aberrations induced by hydrogen peroxide in cultured mammalian cells

BY Hirohisa TSUDA¹⁾

*Biological Research Center, Central Research Institute,
The Japan Tobacco and Salt Public Corporation.
23, Nagoki, Hatano, Kanagawa 257*

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ABSTRACT

Chromosomal aberrations were observed in cultured mammalian cells (CHO-K1 Chinese hamster cells, V79 Chinese hamster cells, Syrian hamster cells, and BALB/c mouse cells) after treatment with hydrogen peroxide (H_2O_2 ; 0.1–0.5 mM or 0.01–0.1 mM) for 3 h. The cytotoxic and clastogenic effects of H_2O_2 were clearly reduced by the addition of catalase. In contrast to the clastogenic potential, H_2O_2 did not enhance the frequency of mutation in V79 cells, whether the marker for mutation was 8-azaguanine resistance or ouabain resistance.

1. INTRODUCTION

Irradiation with x-ray or γ -ray of aqueous solution produces hydrogen peroxide (H_2O_2) (Fricke 1934; Hochanadel 1952; Hart 1952). Near-ultra violet to visible light-irradiation of tryptophan aqueous solution gives rise to substances that have various biological effects including the toxicity for repair-deficient strains of *Escherichia coli* (Webb and Lorenz 1972) and recombinationless mutants of *Salmonella typhimurium* (Yoakum and Eisenstark 1972), and the inhibition of growth in cultured mammalian cells (Stoien and Wang 1974). Recently, one of these biologically active products has been identified as H_2O_2 (McCormick *et al.* 1976).

However, little information is available about the cytogenetic effects of H_2O_2 itself on cultured mammalian cells. Schöneich (1967) reported that chromosomal aberrations were induced by H_2O_2 in strains of ascites tumors in mice. But, his result suffers from some ambiguities in the determination of effective dose of H_2O_2 , since the result is an event in ascites fluid in mouse.

In the present study, H_2O_2 was examined for its potential to induce chromosomal aberrations, and 8-azaguanine or ouabain-resistant mutation in cultured mammalian cells.

2. MATERIALS AND METHODS

Cell culture. Near-term embryos of Syrian hamster and back skin tissue of

¹⁾ Present address: Central Research Institute, The Japan Tobacco and Salt Public Corporation. 6-2, Umegaoka, Midori-ku, Yokohama, Kanagawa 227.

newborn BALB/c mouse were minced by scissors, digested by trypsin (0.25% in Hanks' salt solution), and transferred to primary cultures, respectively. V79 Chinese hamster cells were kindly supplied by Dr. T. Kuroki, (the University of Tokyo), and CHO-K1 Chinese hamster cells were kindly supplied by Dr. H. Katsuta, (the University of Tokyo). Syrian hamster cells, mouse cells and V79 cells were maintained in Eagle's minimum essential medium (MEM; Nissui, Japan) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, USA), 50 $\mu\text{g}/\text{ml}$ of Neomycin (GIBCO, USA), and 1.25 $\mu\text{g}/\text{ml}$ of Fungizone (GIBCO, USA). CHO-K1 cells were cultured in Ham's F-12 medium (Nissui, Japan) instead of MEM. The cells were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in air.

Chemicals. Hydrogen peroxide (H_2O_2 ; Mitsubishi Kagaku, Japan) and catalase (SIGMA, USA, 3,900 U/mg) were dissolved directly in culture medium. 8-Azaguanine (8AG; SIGMA, USA) was dissolved in 0.02N-NaOH solution (2,000 $\mu\text{g}/\text{ml}$). Ouabain (SIGMA, USA) was dissolved in hot-distilled water (20 mM). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Wako, Japan) was dissolved in ethanol (1,000 $\mu\text{g}/\text{ml}$) just before the experiment.

Cytogenetic experiments. The cells (Syrian hamster cells at 2nd passage, BALB/c mouse cells at 2nd passage, CHO-K1 cells, and V79 cells) were seeded into a 75 cm^2 -culture bottle (Falcon, USA) at a density of 1×10^6 cells per bottle. Twenty-four hours after the seeding, the cells were treated with H_2O_2 for 3 h, washed twice with Hanks' balanced salt solution and maintained for 24 h in normal culture medium. For the last 3 h, colcemid (0.1–0.3 $\mu\text{g}/\text{ml}$) was added. The chromosomes were then prepared routinely (Rothfels and Siminovich 1958). The classification of chromosomal aberrations is based on a system proposed by Savage (1976).

Mutation assay. Mutation was assayed with a test system involving forward mutation from 8AG- or ouabain-sensitivity to resistance. V79 cells (1×10^6 cells/bottle) were seeded into a 75 cm^2 -culture bottle. Twenty-four hours after the seeding, the cells were treated with the chemicals (H_2O_2 or MNNG) for 3 h, washed twice with Hanks' salt solution, and detached by 0.25% trypsin (Difco, USA) solution. Immediately after the trypsinization, the cells were reseeded into a 60 mm dish (Falcon, USA). For the mutation assay, 5×10^4 cells per dish were seeded and maintained in normal medium for 48 h. The medium was then replaced with the selection medium (medium containing 20 $\mu\text{g}/\text{ml}$ of 8AG or 1 mM of ouabain). These selection media were replaced twice until fixation. Ten days after the reseeded, the cells were fixed with methanol and stained with Giemsa. For the cell survival experiment, 200 cells per dish were seeded and cultured in normal medium for 7 days. The cells were then fixed and stained. The frequency of mutation was calculated per 10^6 survivors.

Table 1. Chromosomal aberrations induced by H_2O_2 in cultured mammalian cells

Cells	Treatment H_2O_2 (mM)	No. of metaphases counted	Abnormal metaphases (%)	Aberrations in metaphases examined			
				Gaps*	Chromatid breaks	Chromosome breaks	Exchanges* Minutes*
CHO-K1 (Expt. I)	0	100	3	3	0	0	0
	0.1	100	10	10	0	0	0
	0.2	100	22	10	7	0	19
	0.3	100	30	6	5	1	49
	0.5	20	65	5	3	0	14
	1	ND**					16
CHO-K1 (Expt. II)	0	100	2	2	0	0	0
	0.1	100	6	4	1	0	1
	0.2	100	21	10	13	0	14
	0.3	100	40	13	19	3	40
	1	ND**					9
V79	0	100	1	1	0	0	0
	0.1	100	9	7	2	0	2
	0.2	100	15	4	7	0	9
	0.3	6	50	2	3	1	2
	0.5	ND**					
	1	ND**					

* Numbers represent the sum of chromatid- and chromosome-type aberrations.

** Metaphase cells were not detected.

Table 2. Chromosomal aberrations induced by H_2O_2 in cultured mammalian cells

Cells	Treatment H_2O_2 (mM)	No. of metaphases counted	Abnormal metaphases (%)	Aberrations in metaphases examined			
				Gaps*	Chromatid breaks	Chromosome breaks	Exchanges* Minutes*
Syrian hamster	0	100	3	3	0	0	0
	0.1	100	12	12	1	0	2
	0.2	66	25.8	7	13	1	15
	0.3	36	50	11	5	1	3
	0.5	ND**					
	1	ND**					
BALB/c mouse	0	100	5	3	1	0	1
	0.01	100	10	10	8	0	0
	0.02	100	13	13	8	0	1
	0.05	100	27	18	13	6	4
	0.1	50	60	12	13	0	13
	0.2	ND**					
	0.3	ND**					
	0.5	ND**					
	1	ND**					

* Numbers represent the sum of chromatid- and chromosome-type aberrations.

** Metaphase cells were not detected.

Fig. 1. Chromosomal aberrations induced by H_2O_2 in cultured mammalian cells.
a Chromatid exchange (arrow) seen in CHO-K1 cell after treatment with 0.3mM of H_2O_2 .
b Chromatid exchange (arrow) seen in V79 cell after treatment with 0.3 mM of H_2O_2 .
c Chromatid exchange (arrow) seen in BALB/c mouse cell after treatment with 0.1 mM of H_2O_2 .
d Numerous chromosomal aberrations (arrows) seen in BALB/c mouse cell after treatment with 0.05 mM of H_2O_2 .

3. RESULTS AND DISCUSSION

Chromosomal aberrations. Results are shown in Tables 1 and 2. In Chinese hamster cells and Syrian hamster cells, H_2O_2 (0.1–0.5 mM) induced significant amount of chromosomal aberrations including gaps, breaks, exchanges, and minutes. As the dose of H_2O_2 increased, the mitotic index decreased and damages to metaphase chromosomes increased. Especially, H_2O_2 at doses of 0.2 mM and 0.3 mM, induced remarkable exchange-type aberrations. On the other hand, in mouse cells (Table 2), H_2O_2 induced chromosomal aberrations at lower doses (0.01–0.1 mM) than in hamster cells. The frequency of exchange-type aberrations was not so high as that in hamster cells. Typical chromosomal aberrations induced by H_2O_2 are shown in Fig. 1 (a-d).

Reduction of H_2O_2 -effects by catalase. To examine whether the effects of H_2O_2 are reduced by the addition of catalase, 1,000 U/ml of catalase was added to the cells (CHO-K1 cells) from 3 h before H_2O_2 treatment to the end of the treatment. Table 3 shows the result of the cell survival experiment. In the absence of catalase, H_2O_2 caused a decrease in cell survival of about 24% at 0.1 mM and of 100% at doses higher than 0.3 mM. The cytotoxic effect of H_2O_2 , however, was clearly reduced by the addition of catalase. Even at 1 mM of H_2O_2 , survived colonies were observed in the presence of catalase. Table 4 shows the effect of catalase on the clastogenic effect of H_2O_2 . H_2O_2 at 0.3 mM, induced a high rate (30%) of chromosomal aberrations in the absence of catalase. However, in its presence, even at 1 mM, the rate of chromosomal aberrations was very low and almost the same as that of the untreated cultures.

Table 3. *Reduction by catalase of the cytotoxic effect of H_2O_2 in CHO-K1 cells**

Treatment H_2O_2 (mM)	Catalase** (1,000 U/ml)	Relative*** plating efficiency (%)
0	—	100
0	+	100.9
0.1	—	76.5
0.1	+	98.9
0.3	—	0.0
0.3	+	71.6
1	—	0.0
1	+	22.4

* For measurement of plating efficiency, 200 cells were plated in a 60 mm dish. Three hours after the plating, the cells were treated with H_2O_2 at indicated doses for 3 h, washed twice with Hanks, salt solution, and maintained in normal medium for 7 days. The cells were then fixed and stained. Three dishes were used to establish each point.

** Catalase was added to the cells from 3 h before H_2O_2 treatment to the end of the treatment. +; catalase added, —; not added.

*** Plating efficiency of untreated controls; 91.5%.

Table 4. *Reduction by catalase of the chromosomal aberrations induced by H_2O_2 in CHO-K1 cells*

Treatment H_2O_2 (mM)	Catalase* (1,000 U/ml)	No. of metaphases counted	Abnormal** metaphases (%)
0	—	100	3
0	+	100	3
0.3	—	100	30
0.5	—	20	65
0.5	+	100	7
1	—	ND***	
1	+	100	5

* Catalase was added to the cells from 3 h before H_2O_2 treatment to the end of the treatment. +; catalase added, —; not added.

** Percentage of metaphase cells with one or more chromosomal aberrations.

*** Metaphase cells were not detected.

Table 5. *Effect of H_2O_2 on the frequency of 8-azaguanine- or ouabain-resistant mutation in V79 Chinese hamster cells*

Marker for mutation*	Treatment	Survival (%)	No. of mutant colonies	No. of dishes	Mutation frequency/10 ⁶ survivors
8AG	H_2O_2 0 (mM)	97.0	12	10	24.7
	" 0.1	43.3	8	10	37.0
	" 0.2	29.0	4	10	27.6
	" 0.3	6.0	0	10	0.0
	MNNG 0.5 (μ g/ml)	70.0	98	10	280.0
Oua	H_2O_2 0 (mM)	97.0	1	10	2.1
	" 0.1	43.3	2	10	9.2
	" 0.2	29.0	1	10	6.9
	" 0.3	6.0	0	10	0.0
	MNNG 0.5 (μ g/ml)	70.0	64	10	182.9

* 8AG; 8-azaguanine-resistant mutation, Oua; ouabain-resistant mutation.

These results suggest that the clastogenic and cytotoxic effects found in this experiment, are attributable to H_2O_2 .

Mutation experiments. Results are shown in Table 5. The frequency of mutation increased significantly by MNNG which was used as a positive control. However, H_2O_2 induced neither 8AG-resistant mutation nor ouabain-resistant mutation in our experimental conditions.

The data presented here offer unequivocal evidence for the capacity of H_2O_2 to induce chromosomal structural changes in various types of mammalian cells *in vitro*. The result may also present a serious problem for radiation-induced chromosomal aberrations, since H_2O_2 is produced easily by radiation of aqueous

solution as mentioned in the top of this paper.

In contrast to the clastogenic potential, H_2O_2 did not induce significant drug-resistant mutation whether the marker for mutation was 8AG-resistance or ouabain-resistance. At present, it is difficult to explain why H_2O_2 induces chromosomal aberrations without drug-resistant mutation. These results, however, seem to be consistent with the finding that H_2O_2 rapidly inactivates transforming activity of DNA but induces no point mutation in *Bacillus subtilis* (Freese and Freese 1965; Freese 1971).

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