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BIMODAL PATTERN OF KILLING OF CHINESE HAMSTER V79 VARIANT CELLS BY HYDROGEN PEROXIDE

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To elucidate the mechanism of cytotoxicity of H_2O_2, we selected H_2O_2-resistant Chinese hamster V79 cells by single-step selection from a pool of spontaneous variants. The resistant cells showed bimodal sensitivity to H_2O_2 without exhibiting a significantly higher level of the detoxicating enzymes, catalase, glutathione peroxidase and superoxide dismutase. Mode-one and mode-two killing were observed at lower ($<300\,\mu M$) and higher (>2 mM) H_2O_2 concentrations, respectively. Mode-one but not mode-two killing was prevented by iron chelators. Pretreatment with low concentrations of ascorbic acid preferentially enhanced the killing at higher H_2O_2 concentrations. These resistant cells were cross-resistant to t-butyl hydroperoxide and cumene hydroperoxide.

KEY WORDS: Hydrogen peroxide, Cytotoxicity, Resistant Cells, Chinese hamster V79.

Abbreviations: t-BOOH, t-butyl hydroperoxide; CuOOH, cumene hydroperoxide; NBT, nitro blue tetrazolium, MEM, modified Eagle medium; FBS, fetal bovine serum; BME, basal medium Eagle; PBS, phosphate buffered saline; SOD, superoxide dismutase; DETAPAC, diethylenetriaminepentaacetic acid; G-6-PD, Glucose-6-phosphate dehydrogenase.

Hydrogen peroxide is well known for its cytotoxicity and may be implicated in the pathology of various diseases, such as cardiac ischemia, diabetes and cancer.[1] It is the consensus that the primary activating process of H_2O_2 includes a metal-mediated Fenton-type reaction producing ·OH radicals.[1] These radicals induce various cellular lesions: oxidation of proteins and DNA, membrane peroxidation, changes in nucleotide levels, increase in cytosolic Ca^{2+}, and mitochondria damage. However, none of these lesions has been well established as being responsible for cell death.[2,3,4]

To elucidate the mechanism of cytotoxicity of H_2O_2, several investigators have used H_2O_2-resistant cells.[5,6,7] They have usually been selected by a stepwise increase in H_2O_2 and devided into two groups. Cells in the first group have increased activity of detoxicating enzymes such as catalase and glutathione peroxidase,[5,6] while those in the second group have minor changes in catalase activity.[7] Resistance in the first group might be due to adaptation to a high H_2O_2 concentration.

We selected H_2O_2-resistant Chinese hamster V79 cells by single-step selection from a pool of spontaneous variants and characterized the resistant cells. These cells

[1] Correspondence

showed bimodal sensitivity to H_2O_2 without exhibiting significantly higher levels of detoxicating enzymes. Furthermore, these resistant cells were cross-sensitive to t-BOOH and CuOOH but not to menadione. These cells might be useful for clarifying the mechanism of cytotoxicity of hydroperoxides.

MATERIALS AND METHODS

Chemicals

Xanthine oxidase, catalase, glutathione reductase, xanthine, o-phenanthroline, glutathione and NBT were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Desferal was purchased from Japan-Chiba Geigy Co. (Hyogo, Japan).

Isolation of Chinese Hamster Variants

Chinese hamster V79 cell were a gift from Dr. T. Kuroki (Institute of Medical Science, University of Tokyo) and were cultured in MEM (Nissui Pharmaceutical Co., Tokyo, Japan) with 10% FBS in a humidified 5% CO_2 incubator. Logarithmically growing V79 cells were seeded of 5×10^5 cells/100-mm Petri dish (Nunc, Roskilde, Denmark) in 8 ml of MEM supplemented with 10% FBS. At 24 hr after seeding, the cells were exposed once to 4 mM H_2O_2 in BME without serum for 1 hr at 37°C, washed with PBS, trypsinized, and cultured for a week. The fraction surviving at 4 mM H_2O_2 was 8.7×10^{-5}. Three large colonies visible in the dish were isolated, and assayed for plating efficiency after exposure to 4 mM H_2O_2. A single colony showed resistance to H_2O_2 and was characterized further. The resistant cells have a growth rate similar to that of the parent cells, keeping the resistant phenotype for more than 6 months.

Colony-forming Assay

The relative plating efficiencies in the presence of different concentrations of drugs were determined as the ratio of the number of colonies at a given drug concentration to that obtained in the control culture in the absence of any drug. Cells were seeded at 5×10^5/60-mm Petri dish in 4 ml of MEM supplemented with 10% FBS. On the next day, cells were exposed to drugs in BME without serum for 1 hr at 37°C, washed with PBS, and trypsinized, and 200 to 10,000 cells were seeded in 60-mm Petri dishes with 4 ml of MEM containing 10% FBS and cultured for 6 days. The dishes were fixed with methanol and stained with 7% Giemsa solution. The colonies (>50 cells/colony) were counted under a dissecting microscope. Groups of four replicate dishes were used to assess the effect of drugs on plating efficiency.

Preparation of Substrate for Enzyme Assay

Cells (1×10^7) were harvested, washed 2 times with PBS without Ca^{2+} and Mg^{2+} and sonicated in 1 ml of 50 mM potassium phosphate buffer (pH 7.4). The sonicates were centrifuged at 15,000 rpm for 20 min at 4°C and the cell supernatants were used for enzyme analysis. The protein content of the cells was determined by the Lowry method.

Enzyme Assay

Glutathione peroxidase activity was assayed according to the method of Lawrence and Burk.[8] The reaction mixture contained 0.1 ml of 0.5 M potassium phosphate buffer (pH 7.4), 0.1 ml of cell supernatant, 0.1 ml of 10 U/ml glutathione reductase, 0.1 ml of glutathione, 0.1 ml of 1.5 mM NADPH, 0.1 ml of 10 mM EDTA, 0.1 ml of 10 mM NaN_3, and 0.1 ml of 12 mM t-BOOH or 0.15 mM H_2O_2 as a substrate (total volume, 1 ml). Activity of the enzyme was expressed as nmol of NADPH/min/mg protein or 10^6 cells.

Catalase activity was assayed according to the method described by Aebi.[9] The reaction mixture contained 0.1 ml of 0.5 M potassium phosphate buffer (pH 7.0), 0.1 ml of 10 mM H_2O_2, and 0.1 ml of cell supernatant (total volume, 1.0 ml). One unit of activity was defined as the amount of protein required to decompose 1 μmol H_2O_2 per min at room temperature.

SOD activity was assayed according to the method of Spitz and Oberley.[10] The reaction mixture contained 0.1 ml of 0.5 M potassium phosphate buffer (pH 7.8), 0.1 ml of 10 mM DETAPAC, 1 U of catalase, 0.1 ml of 6.6×10^{-4} M NBT, 0.1 ml of 1 mM xanthine, 15 mU of xanthine oxidase, and 0.1 ml of cell supernatant (total volume, 1 ml). The reference rate was defined as the rate of increase in absorbance at 560 nm without cell supernatant. One unit of activity was defined as the amount of protein necessary to reduce the reference rate by 50%.

G-6-PD activity was assayed according to Holliday and Tarrant.[11] The reaction mixture contained 0.8 ml of 55 mM Tris-HCl (pH 7.8), 3.3 mM $MgCl_2$, 50 μl of 12 mM $NADP^+$, 50 μl of 0.1 M glucose-6-phosphate and 0.1 ml of sample supernatant (total volume, 1.0 ml). Increase in absorbance at 340 nm resulting from the reduction of $NADP^+$ was measured. One unit was defined as the amount required to reduce of 1 μmol of $NADP^+$ per min at 30°C.

RESULTS

Comparison of Killing of Parent and Resistant (Hpr4) Cells by Hydrogen Peroxide

Responses of the parent and resistant (Hpr-4) cells to H_2O_2 are shown in Figure 1-a. Survival of the parent cells as a function of H_2O_2 concentration was consistent with that reported previously[5,6]. On the other hand, the survival curve of Hpr-4 cells showed a broad shoulder of resistance at concentrations up to 5 mM, accompanied by a small dip below 300 μM, reflecting two modes of killing of Hpr-4 cells by H_2O_2. The time course of killing of Hpr-4 cells by 100 μM (mode-one killing) and 2.5 mM H_2O_2 (mode-two killing) is shown in Figure 1-b. The killing was almost linear with time up to 1 hr. Since the apparent killing rates at 100 μM and at 2.5 mM H_2O_2 were similar for the two types of killing, H_2O_2 at 100 μM might kill Hpr-4 cells 20 times more efficiently than at 2.5 mM.

Level of Activity of Enzymes Related to Active Oxygen Species in the H_2O_2-resistant Cells

In previous studies, the activity of catalase and/or SOD of H_2O_2-resistant cells in the first group was higher than that of their parent cells,[5,6] but this is not the case in the second group.[7] We compared the levels of detoxicating as well as activating

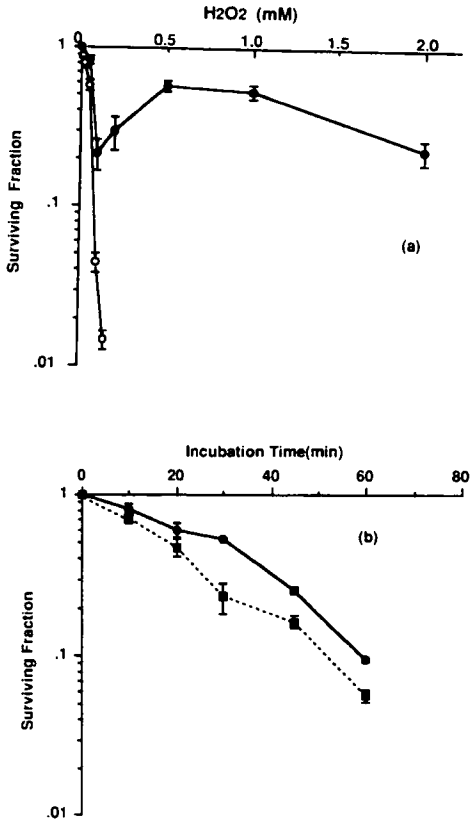

FIGURE 1 The relative plating efficiencies of Chinese hamster V79 parent and H_2O_2-resistant (Hpr-4) cells treated with H_2O_2 at 37°C. a) Effect of various concentrations of H_2O_2 on the cytotoxicity. —○— parent, —●— Hpr-4 cells. Each point represents the mean of 4 dishes; bars, SE. b) Time course of relative plating efficiencies of Hpr-4 cells treated with low and high concentrations of H_2O_2. —●— 100 μM, ···■··· 2.5 mM H_2O_2. Each point represents the mean of 4 dishes; bars, SE.

enzymes of Hpr-4 cells with those of the parent cells. Table I shows the activity of catalase, total SOD, glutathione peroxidase and G-6-PD on a per mg of protein or per 10^6 cells basis as measured in lysates of both the parent and resistant cells. The level of the activity of the detoxicating enzymes in Hpr-4 cells is not significantly higher than that in the parent cells. Therefore, the abnormal survival curve of Hpr-4 cells cannot be ascribed to changes in enzyme activity for detoxication.

Effect of Metal Chelators on the Cytotoxicity of Hydrogen Peroxide

H_2O_2-induced cytotoxicity so far reported has been inhibited by metal chelators such as o-phenanthroline and Desferal, indicating a Fenton-type activation of H_2O_2 into hydroxyl radicals.[1] The effects of these metal chelators on the survival of Hpr-4 cells exposed to H_2O_2 are shown in Figure 2-a and b. The dip in the survival curves in the absence of the metal chelators ($\sim$300 μM) disappeared in the

TABLE I
Enzyme activities related to metabolism of active oxygen species in parent and H_2O_2-resistant Chinese hamster V79 cells

		V79		Hpr-4	
SOD	(Exp.)	(U/mg protein)	($U/10^6$cells)	(U/mg protein)	($U/10^6$cells)
	1	22.7	0.98	21.9	1.21
	2	16.1 ± 0.5	1.01 ± 0.03	18.2 ± 0.4	0.90 ± 0.01
Glutathione Peroxidase	(Substrate)	(nmol/mg protein)	(nmol/10^6cells)	(nmol/mg protein)	(nmol/10^6cells)
	t-BOOH	3.46 ± 0.45	0.216 ± 0.028	3.88 ± 0.67	0.250 ± 0.043
	H_2O_2	4.58 ± 0.40	0.285 ± 0.025	5.80 ± 0.023	0.342 ± 0.015
Catalase		($\times 10^{-3}$U/mg protein)	($\times 10^{-4}U/10^6$cells)	($\times 10^{-3}$U/mg protein)	($\times 10^{-4}/10^6$cells)
		5.21 ± 0.02	2.61 ± 0.12	5.48 ± 1.18	3.58 ± 0.79
G-6-PD		(nmol/mg prot./min)	(nmol/10^6cells/min)	(nmol/mg prot./min)	(nmol/10^6cells/min)
		69.3 ± 1.6	3.47 ± 0.08	73.5 ± 9.4	4.83 ± 0.62

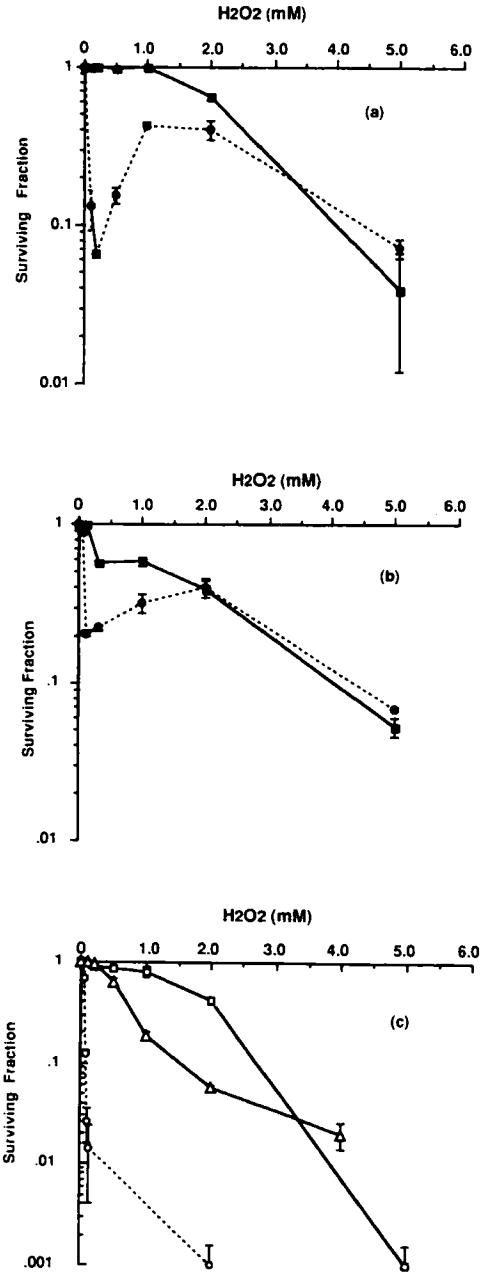

FIGURE 2 Effects of metal chelators on the plating efficiencies of the parent and Hpr-4 cells treated with various concentrations of H_2O_2. a) Effects of o-phenanthroline on Hpr-4 cells —■—, ··●··, with and without o-phenanthroline (50 μM), respectively. b) Effects of pretreatment with Desferal (5 h) on Hpr-4 cells —■—, ··●··, with and without Desferal (200 μM), respectively. c) Effects of o-phenanthroline and pretreatment with Desferal on the parent cells. —□—, ··○·· with and without o-phenanthroline, respectively; —△—, with Desferal. Each point represents the mean of 4 dishes; bars, SE.

curve in their presence. However, these chelators did not alter the toxicity at higher concentrations of H_2O_2 (>2 mM). In the parent V79 cells, the toxicity at lower concentrations of H_2O_2 was prevented by the presence of o-phenanthroline and Desferal, while the toxicity at higher concentrations of H_2O_2 (>2 mM) in the presence of o-phenanthroline (Figure 2-c) was similar to that of Hpr-4 cells. Desferal was less effective than o-phenanthroline in inhibiting the cytotoxicity to the parent cells by higher concentrations of H_2O_2. At any rate, these chelators did not protect either the parent or the Hpr-4 cells from the toxic effects of H_2O_2 at higher concentrations (>2 mM).

Effect of Pretreatment with Ascorbic Acid on the Cytotoxicity of Hydrogen Peroxide

Ascorbic acid, an anti-oxidant used to shift the cellular redox equilibrium, has been reported to potentiate the cytotoxic effects of H_2O_2 at 0°C but not at 37°C.[12] Figure 3 shows the toxic effects of H_2O_2 on the parent and Hpr-4 cells after treatment with various concentrations of ascorbic acid. The cytotoxicity of both the parent cells and Hpr-4 cells exposed to 50 μM H_2O_2 at 37°C was little potentiated by pretreatment with 1 mM ascorbic acid, in accordance with the findings of Jonas *et al.*,[12] whereas the toxicity for Hpr-4 cells at 1.5 mM H_2O_2 was drastically enhanced by pretreatment with more than 20 μM ascorbic acid (the surviving fraction at 1 mM ascorbic acid was 0 and could not be shown in Figure 3).

Cross-sensitivity to Other Oxidants

The sensitivity of H_2O_2-resistant cells to other oxidants was tested. As shown in Figure 4, Hpr-4 cells were also resistant to t-BOOH and CuOOH and showed a bimodal response at least to t-BOOH. However, the resistant cells were sensitive to menadione (data not shown). Although a Fenton-type reaction producing reactive radicals might be involved in the killing of cells by hydroperoxides and quinones,[1] the resistance of Hpr-4 cells was limited to hydroperoxides without extending to quinones.

DISCUSSION

We isolated H_2O_2-resistant Chinese hamster V79 cells by single-step selection from a pool of spontaneously generated variants. The resistant cells showed bimodal sensitivity to H_2O_2. Similar bimodal killing by H_2O_2 has been reported for *Escherichia coli*;[13] however, this is the first observation for mammalian cells.

Since the H_2O_2-resistant (Hpr-4) cells isolated in the present study have levels of detoxicating enzymes similar to those of the parent cells, they could be assigned to the group of resistant cells with only mionor changes in antioxidant enzyme levels. However, the possibility cannot be excluded that the intracellular compartmentation of scavenging enzymes might be altered, without an increase in total enzyme activity, so as to protect cells from oxidative stress.

Based on the results for *E. coli*,[13] we can tentatively define the two modes of killing by H_2O_2 as follows: Mode-one killing is mediated by Fe^{2+} ions and is inhibited by Desferal and o-phenanthroline. Proximate reactants of mode-one killing are believed to be hydroxyl radicals produced by a Fenton-type reaction,

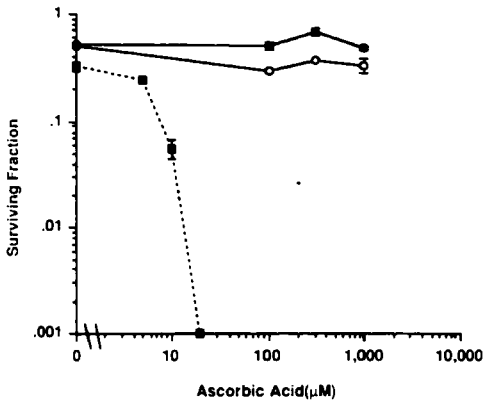

FIGURE 3 Effects of pretreatment with different concentrations of ascorbic acid on the plating efficiencies of the parent and Hpr-4 cells exposed to H_2O_2. —○—, —●—, parent and Hpr-4 cells, respectively, exposed to 50 μM H_2O_2, ··■··, Hpr-4 cells exposed to 1.5 mM H_2O_2. Each point represents the mean of 4 dishes; bars, SE.

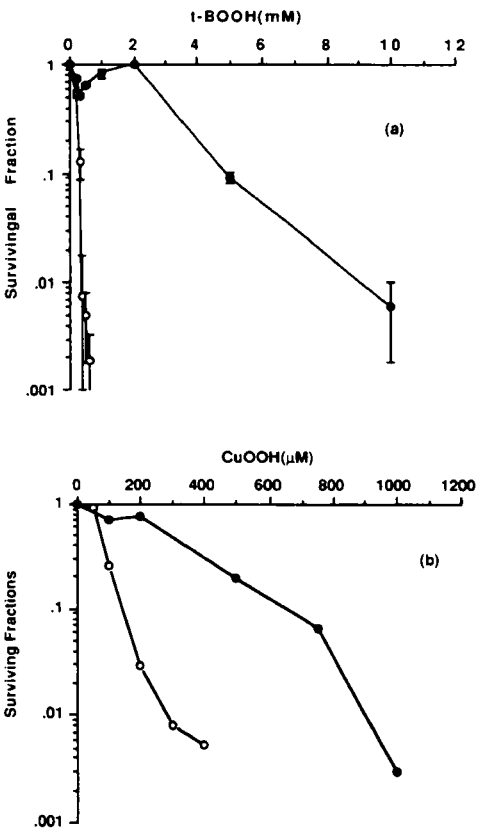

FIGURE 4 The relative plating efficiencies of the parent and Hpr-4 cells treated with t-BOOH (a) and CuOOH (b). —○— parent cells, —●— Hpr-4 cells. Each point represents the mean of 4 dishes; bars, SE.

while mode-two killing is not mediated by Fe^{2+} ions and not inhibited by Desferal and o-phenathroline. Mode-one killing in *E. coli* is slight in wild-type cells and enhanced in mutants with DNA repair deficiencies. It requires active metabolism generating the reduced nicotinamide nucleotides. Mode-two killing is dominant in wild-type *E. coli* and does not require active metabolism. In spite of the above statements about mode-two killing, its mechanism in *E. coli* remains obscure.

In Chinese hamster V79 cells, killing of the parent and Hpr-4 cells by low concentrations of H_2O_2 ($<300\,\mu M$) was inhibited by Desferal and o-phenanthroline (Figure 2), indicating the contribution of Fe^{2+} ions. Thus, the killing at low concentrations of H_2O_2 can be identified as mode-one killing. At moderate concentrations of H_2O_2 ($300\,\mu M \sim 2\,mM$), killing of the parent cells was still mostly mode-one killing, since it was inhibited by o-phenanthroline. On the other hand, killing of Hpr-4 cells by these concentrations of H_2O_2 was partly inhibited by Desferal and by o-phenanthroline, indicating summation of both mode-one and mode-two killing. At higher concentrations of H_2O_2 ($>2\,mM$), killing of Hpr-4 cells was not inhibited by either Desferal or o-phenanthroline and was identified as mode-two killing. In contrast to the parent cells, Hpr-4 cells are characterized by slight or little contribution of mode-one killing at moderate and higher concentrations of H_2O_2. The rate of killing of Hpr-4 cells as related to H_2O_2 concentration at $100\,\mu M$ H_2O_2 was about 20 times higher than that at $2.5\,mM$ H_2O_2 (Figure 1-b). The difference in the efficiency of killing may be due to different proximate reactants in the two modes of killing by H_2O_2 or to changes in intracellular iron distribution for the Fenton type reaction induced by high concentrations of H_2O_2.

Killing of the parent cells in the presence of o-phenanthroline at higher concentrations of H_2O_2 should be ascribed to mode-two killing (Figure 2-c). Inhibition of killing of the parent cells by Desferal at moderate and higher concentrations of H_2O_2 was much more limited than that by o-phenanthroline. This might be due to the difference between Desferal and o-phenanthroline in the ability to permeate the plasma membrane, intracellular distribution due to hydrophobicity or stability of chelating complexes with Fe^{2+}/Fe^{3+}. Without o-phenanthroline, a slight contribution of mode-two killing in the parent cells might be expected from the slope of the survival curve at moderate H_2O_2 concentrations ($300\,\mu M \sim 2\,mM$), which was different from that at lower H_2O_2 concentrations and similar to that with o-phenanthroline at high concentrations ($>2\,mM$), already ascribed to mode-two killing. Thus, the killing of the parent cells at moderate and higher H_2O_2 concentrations could be the result of the superposition of a small amount of mode-two killing on the prevailing mode-one killing. The possibility that a subpopulation of resistant cells generated in the parent cells contributed to the slight mode-two killing was excluded; cells recloned from the parent cells also showed a similar different slope in the survival curves (data not shown). Thus, we should emphasize that mode-two killing was a normal physiological process in Chinese hamster V79 cells. Suppression of mode-one killing in the parent cells should reproduce the response of Hpr-4 cells. Similar assignment of modes of killing can be expected for the parent and Hpr-4 cells exposed to t-BOOH (Figure 4-a).

Iron metabolism usually depends on reducing equivalents normally generated by cellular metabolism.[12, 14] In order to study the role of reducing equivalents in mode-one killing of Hpr-4 cells by H_2O_2, we investigated potentiation by ascorbic acid of mode-one killing of Hpr-4 cells at $1.5\,mM$ H_2O_2. Pretreatment of Hpr-4 cells with a low concentration of ascorbate ($<20\,\mu M$) was enough to potentiate the

killing by H_2O_2 (1.5 mM), whereas the killing of both Hpr-4 cells and the parent cells by low concentrations of H_2O_2 (50 μM) was little enhanced by 1 mM ascorbic acid. Our interpretation of these results is that the oxidized form of iron was reduced to Fe^{2+} by ascorbate, thereby activating the unreacted higher concentrations of H_2O_2 to hydroxyl radicals. This interpretation is consistent with the findings that pretreatment with Desferal abolished the action of ascorbate[12] and that the cytotoxicity of the parent cells exposed to 1.5 mM H_2O_2 for 10 min was potentiated by pretreatment with ascorbate (data not shown). Therefore, suppression of mode-one killing of Hpr-4 cells at moderate concentrations of H_2O_2 may be due to insufficient supply of cellular reducing equivalents, at least at certain critical sites. Although NADPH is proposed as the reducing equivalent for *E. coli*,[14] there was no difference between the parent and Hpr-4 cells in the activity of G-6-PD, which should determine most of the cellular level of NADPH (Table I). Changes in intracellular distribution of NADPH in Hpr-4 cells or contribution of other reducing equivalents would be important determinants.

Recently, the killing of central neural cells by t-BOOH was shown to be prevented by an oncogene product, BCL-2, which is reported to be located in the mitochondria inner membrane.[15] Since H_2O_2 and other organic hydroperoxides may share a common mechanism of cytotoxicity, it is important to know the function of BCL-2 in cytotoxicity of hydroperoxides for fibroblasts.

Several questions must be answered to determine the critical target for two modes of killing: whether Hpr-4 cells are deficient in certain cellular reducing equivalents; what cellular lesions induced in Hpr-4 cells are fewer than those in the parent cells; whether enhanced killing by ascorbic acid accompanies certain enhanced lesions; whether certain cellular lesions were repaired more efficiently in Hpr-4 cells, etc. Direct measurement of the amount of hydroxyl radicals generated from H_2O_2 in the parent and Hpr-4 cells in the absence or presence of ascorbic acid would be the first step to answering these questions.

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