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Isolation and Preliminary Characterization of a Chinese Hamster Ovary Cell Line with High-Degree Resistance to Hydrogen Peroxide

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ABSTRACT. We have isolated and conducted preliminary characterization of a cell line derived from the Chinese hamster ovary cell line AA8, which we have designated AG8 and which is highly resistant to the cytotoxic effects of H_2O_2 (~17-fold when the H_2O_2 treatment was at 37°; ~11-fold when the H_2O_2 treatment was at 4°). AG8 cells were moderately (but significantly; $P < 0.05$) cross-resistant to $CdCl_2$ (~4-fold), $NaAsO_2$ (~2.3-fold), *t*-butyl hydroperoxide (~2.9-fold), cumene hydroperoxide (~3-fold), menadione (~1.7-fold) and $HgCl_2$ (~1.5-fold), but were not significantly cross-resistant to hyperthermia (43°), 254 nm UV light, ^{137}Cs γ-rays, and 42-MeV ($p \rightarrow Be^+$) fast neutrons. As regards their biochemical status, AG8 and AA8 cells contain similar non-protein sulfhydryl levels per milligram of protein. Catalase activity (assessed by both spectrophotometry and polarography) was significantly higher in AG8 than in AA8 cells irrespective of whether enzyme activity was expressed per 10^6 cells (~3.6-fold increase) or per milligram of protein (~1.6-fold increase). AG8 cells also exhibited significantly greater glutathione reductase activity than wild-type cells when the data were expressed per 10^6 cells (~2.9-fold) or per milligram of protein (~1.3-fold). Glutathione peroxidase activity was immeasurably low in both cell lines. The susceptibility of the two cell lines to H_2O_2-mediated generation of DNA single-strand breaks (as measured by alkaline elution) indicated a slightly (~1.5 fold) decreased yield in the resistant AG8 cell line. The two cell lines repaired these breaks with similar kinetics. In contrast, no measurable induction of DNA double-strand breaks (as measured by pulsed-field gel electrophoresis) was apparent in either cell line after survival-curve range concentrations of H_2O_2. On the basis of these data, it appears that the AG8 phenotype involves two previously identified resistance mechanisms, namely an adaptive component that may or may not involve increased antioxidant capacity, and a second component that does involve increased antioxidant (primarily catalase) capacity. BIOCHEM PHARMACOL 51;8:1021–1029, 1996.

KEY WORDS. hydrogen peroxide-resistant CHO cells; cytotoxicity; DNA damage

H_2O_2 is a potentially cytotoxic metabolite present in all aerobic mammalian cells [1, 2]. Although cellular antioxidant defenses normally protect against the damaging effects of ROIs§ derived from H_2O_2, under some conditions such species may be generated at a high rate, and detrimental biological effects can result. Evidence in the literature indicates that mammalian cells can develop tolerance to H_2O_2 [3]. The development of tumor-cell resistance to an array of ROI-producing antitumor drugs, associated with an increased antioxidant capacity, is a clinical problem that has prompted active investigation into the mechanisms by which cells acquire such resistance. Spitz and colleagues [4–7] have developed several H_2O_2-resistant cell lines that provide insight into some aspects of the acquisition of the ROI-resistant phenotype. When cells are exposed to a relatively high concentration of H_2O_2, they develop a stable resistance that is accompanied by significant changes in the levels of antioxidant enzymes, most notably catalase (EC 1.11.1.6). In contrast, H_2O_2-resistant CHO-cell variants with relatively small alterations in catalase activity have been obtained following adaptation of a cell culture to gradually increasing concentrations of the oxidant [8]. In these variants, the H_2O_2-resistant phenotype only partially depends on the increased efficiency of detoxification mechanisms [8], is accompanied by minimal alterations in the levels of the NPSH pool [9], and is not stable [8]. From

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§ *Abbreviations:* ROIs, reactive oxygen intermediates; CHO, Chinese hamster ovary; NPSH, non-protein sulfhydryl; WT, wild-type; AT, 3-amino-1,2,4-triazole; GSH, reduced glutathione; CHP, cumene hydroperoxide; GSSG, oxidized disulfide of GSH; BSO, L-buthionine-[S,R]-sulfoximine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SSBs, single-strand breaks; SSFs, strand-scission factors; DSBs, double-strand breaks; and TBHP, *t*-butyl hydroperoxide.

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one of these variants, we have isolated a number of cell lines with various degrees of resistance to H_2O_2. We here report the preliminary characterization of one of these cell lines, which we named AG8 and which is extremely resistant to the cytotoxic effects of H_2O_2.

MATERIALS AND METHODS

Cell Culture and H_2O_2 Treatments

CHO WT AA8 and H_2O_2-resistant AG8 cells were grown in monolayer culture in McCoy's 5A medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Seralab, Sussex, U.K.), penicillin (50 U/mL), and streptomycin (50 μg/mL), at 37° in T-75 tissue culture flasks (Corning Glass Works, Corning, NY) gassed with an atmosphere of 95% air–5% CO_2.

All H_2O_2 treatments were made in 2 mL of Saline A, and solution changes were performed gently to avoid cell detachment. The cytotoxicity of H_2O_2 depends on the cell density [10]; a constant cell density at the H_2O_2 treatment stage was achieved by pre-plating 2.5×10^5 AA8 cells into 60-mm tissue culture dishes and incubating them for 14 hr at 37°, during which time their number increased to ~4×10^5. Since AG8 cells have a slower rate of growth, they were seeded at 3.5×10^5 cells/60-mm tissue culture dish.

Isolation of H_2O_2-Resistant AG8 Cells

AG8 cells were isolated from a culture of H_2O_2-resistant variant V850 cells that had been developed in our laboratory [8]. V850 cells were treated with 1.5 mM H_2O_2 for 1 hr at 37°, and 2 days later were detached by exposure to trypsin and seeded at appropriate dilutions in 30-mm tissue culture dishes. After 12 days, the dishes were examined under a microscope, and those containing a single colony were processed further. These colonies were isolated using a cloning ring and expanded in fresh growth medium before being tested for their resistance to H_2O_2. One of these clones, which demonstrated a relatively high degree of resistance to H_2O_2, was named AG8 and was chosen for further characterization.

Catalase, Glutathione Reductase and Glutathione Peroxidase Activity

Cells were seeded at a density of 2×10^6 cells/T-75 flask; after 24 hr of growth they were harvested by exposure to 1% trypsin for 5 min at 4°. Cell pellets obtained after centrifugation at 400 *g* for 5 min were washed twice and resuspended in Saline A. The cell suspensions were placed on ice and disrupted by sonication three times for 15 sec at 20 W (using a Branson Sonifier). The resulting homogenates were centrifuged for 5 min at 18,000 *g* at 4°. Catalase activity was assayed spectrophotometrically by the method of Aebi [11], which involves monitoring at 240 nm the disappearance of H_2O_2 (10 mM) following the addition of the cell-free supernatant fraction. The enzymatic activity was determined by reference to a standard curve constructed for bovine liver catalase (Sigma, St. Louis, MO), and was expressed in Sigma Units (1 Sigma Unit decomposes 1 μmol H_2O_2/min at pH 7 and 25°). Catalase activity in the supernatant was also assayed polarographically by the method of Del Rio *et al.* [12], which involves monitoring oxygen evolution (using a Clark electrode) from an H_2O_2 (10 mM)-containing solution following the addition of the supernatant fraction.

In those experiments involving the catalase inhibitor AT (CAS 61-82-5) (Sigma), two different protocols were used. When the cells were to be assayed for catalase activity, they were set in T-75 flasks as described above, incubated for 24 hr at 37°, and then treated with 10 mM AT for 6 hr at 37°. When the cells were to be screened for H_2O_2-mediated cytotoxicity, they were set at 2.5×10^5 cells/60-mm tissue culture dish and treated with 10 mM AT for the final 6 hr of a 24-hr incubation at 37°.

GSH reductase (EC 1.6.4.2) activity was estimated by the method of Eklöw *et al.* [13], which involves monitoring at 340 nm the disappearance (by oxidation) of NADPH. GSH peroxidase (EC 1.11.1.9) activity was estimated by the method of Prohaska and Gaunter [14], which again involves monitoring at 340 nm the disappearance of NADPH in the presence of a cell-free extract, this time in a reaction scheme that couples the GSH peroxidase-mediated GSH-dependent reduction of CHP and the GSH reductase-mediated NADPH-dependent reduction of GSSG. Proteins were assayed as described by Lowry *et al.* [15], using bovine serum albumin as a standard.

In those experiments involving GSH depletion, cells were incubated for 16 hr at 37° with the γ-glutamyl-cysteine synthetase inhibitor BSO (CAS 83730-53-4) (Sigma) at a concentration of 7.5 μM. NPSH levels were determined using the DTNB method, as described previously [9].

NAD^+ and ATP Levels

Monolayer cultures were washed twice with ice-cold PBS, and 1 mL of ice-cold 2.5% perchloric acid was added to each plate. After a 10-min incubation at ice-bath temperature (4°), the solution was removed and the extraction procedure was repeated. Cell extracts were neutralized with K_2CO_3, and the precipitate was removed by centrifugation. The resulting nucleotide-containing aqueous solutions were filtered through 0.22 μm pore size microfilters and analyzed for their NAD^+ and ATP content by reversed-phase high performance liquid chromatography using a Supelcosil LC-18 column (Supelco, Bellefonte, PA). The chromatographic apparatus and conditions have been described previously [16].

Cytotoxicity

Cytotoxicity was determined by a conventional clonogenic survival assay. After treatment, appropriate dilutions of cells (selected to give ~100 viable cells/dish) were plated in

triplicate into 60-mm tissue culture dishes and incubated at 37° for 8 days. The resulting colonies were stained with 5% gentian violet-ethanol and counted visually. Surviving cells were defined as those that produced colonies of 50 cells or greater. The surviving fraction was calculated as the ratio of the plating efficiencies of treated versus untreated control cells.

Measurement of DNA SSBs

Cells were labeled overnight with [*methyl*-^{14}C]thymidine (0.05 μCi/mL) and incubated for a further 6 hr in medium containing unlabeled thymidine (1 μg/mL). The cells were exposed to H_2O_2 for 30 min at 37° and removed from the dishes by exposure to 1% trypsin for 5 min at 4°. DNA SSBs were determined by alkaline elution essentially as described by Kohn [17] with minor modifications [10]. The eluted DNA and the DNA retained on the membrane were assayed by liquid scintillation counting. SSFs were calculated from the resulting DNA-elution profiles as described previously [10].

Measurement of DNA DSBs

Cells were labeled with [*methyl*-^{14}C]thymidine and treated with H_2O_2 as detailed above. DNA DSBs were determined by pulsed-field gel electrophoresis as described previously [18]. Briefly, cells were formed into agarose plugs (low-melt; Bio-Rad, Richmond, CA) at 4° and digested with lysis buffer [0.5 M EDTA, 1% Sarkosyl, 1 mg/mL proteinase K (EC 3.4.21.14); pH 9] for 1–2 hr at 4° then at 45° for 20 hr. The DNA in the plugs was separated in a 0.5% agarose gel (chromosomal grade; Bio-Rad) in 0.5× TAE buffer (44.5 mM Tris base, 34.5 mM sodium borate, 1 mM Na_2EDTA; pH 7.2). The gel was run for 20 hr at 25° on a CHEF-DR II system (Bio-Rad) operating at 1.21 V/cm with a switch time of 75 min. The distribution of radioactivity in the gel was determined by cutting portions of the gel corresponding to the DNA in the well and the lane (as identified by ethidium bromide staining) and melting them in glass scintillation vials in the presence of 50 μl of 1 M HCl, followed by liquid scintillation counting.

Flow-Cytometric Analysis of DNA Content

Cells were fixed in 60% ethanol and stained with propidium iodide. DNA histograms were obtained using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CellFIT software.

RESULTS

Biological Characterization and Resistance to H_2O_2

The isolation of the H_2O_2-resistant AG8 cell line was accomplished as described in Materials and Methods. The biological characteristics of the AG8 line were markedly different from those of the WT AA8 line. During logarithmic growth, the two cell lines exhibited mean generation times of ~18 hr (AA8) and ~25.5 hr (AG8). The two cell lines also showed marked morphological differences. Among other things, AG8 cells were larger than AA8 cells, which was also reflected in their ~2.3-fold greater protein content (Table 1). AG8 cells also showed a slight (although non-significant) increase in total DNA content compared with AA8 cells, as evidenced by flow-cytometric analysis (Table 2). In addition, AG8 cells were more rounded than AA8 cells.

The resistant phenotype was first characterized by comparing the effect of a 30-min exposure to H_2O_2 (at 37°) on the survival of AA8 and AG8 cells (Fig. 1A). AG8 cells were highly resistant to the cytotoxic effects of H_2O_2, exhibiting a high (~20%) survival after 20 mM oxidant, a concentration that would have killed essentially all of the AA8 cells. The concentrations of H_2O_2 required to reduce survival by 50% (IC_{50}) in WT and resistant cells were 0.078 ± 0.002 and 1.33 ± 0.05 mM, respectively (Table 3). Thus, there was an ~17-fold difference in sensitivity between the two cell lines at the IC_{50} level.

TABLE 1. Biochemical features of wild-type AA8 and peroxide-resistant AG8 cells

	per 10^6 cells		per mg of protein	
	AA8	AG8	AA8	AG8
Protein content* (μg)	152 ± 10	351 ± 20		
ATP† (nmol)	3.65 ± 0.33	8.07 ± 0.85	24.0 ± 1.9	23.0 ± 1.9
NAD† (nmol)	0.99 ± 0.09	2.46 ± 0.31	6.5 ± 0.5	7.0 ± 0.6
Catalase (Sigma U)	4.41 ± 0.44‡	15.8 ± 1.6‡	29.0 ± 2.9‡	45.0 ± 4.5‡
	4.01 ± 0.38§	14.6 ± 1.2§	26.4 ± 2.0§	41.6 ± 4.0§
Total NPSH‖ (nmol)	6.6 ± 0.4	14.1 ± 1.4	42.9 ± 3.8	40.2 ± 4.7
GSH reductase¶	54.5 ± 4.2	159 ± 13	358 ± 28	452 ± 35

Values are the means ± SEM of at least 3 separate experiments.

* As measured by the method of Lowry *et al.* [15].

† As measured by the method of Stocchi *et al.* [16].

‡ As measured by the method of Aebi [11].

§ As measured by the polarographic method by Del Rio *et al.* [12].

‖ As measured by the DTNB method.

¶ Expressed in mU, where 1 mU reduces 1 nmol of oxidized GSSG/min at pH 7.6 and 25°, as measured by the method of Eklöw *et al.* [13].

TABLE 2. Flow-cytometric analysis of the DNA content of AA8 and AG8 cells

Parameter	AA8	AG8	Ratio
(G_1) peak channel	218 ± 17*	238 ± 31*	1.09
(G_2 + M) peak channel	413 ± 33	446 ± 57	1.08

* Mean ± SEM of 4 separate experiments.

Figure 1B shows the results of experiments similar to those reported in Fig. 1A except that the H_2O_2 treatments were performed at 4°. As previously reported by us [10] and by others [19], H_2O_2 was less cytotoxic at 4° than at 37°. The IC_{50} values in WT and resistant cells were 1.17 ± 0.11 and 12.7 ± 1.3 mM, respectively (Table 3). AG8 cells were significantly (~11-fold at both IC_{50} and IC_{90}) resistant to H_2O_2 ($P < 0.05$) compared with AA8 cells under these experimental conditions. The decreased degree of resistance at 4° compared with that measured at 37° appears to reflect a greater shift in the H_2O_2 sensitivity of the parental line on changing the exposure temperature, the AG8 survival curve moving relatively little.

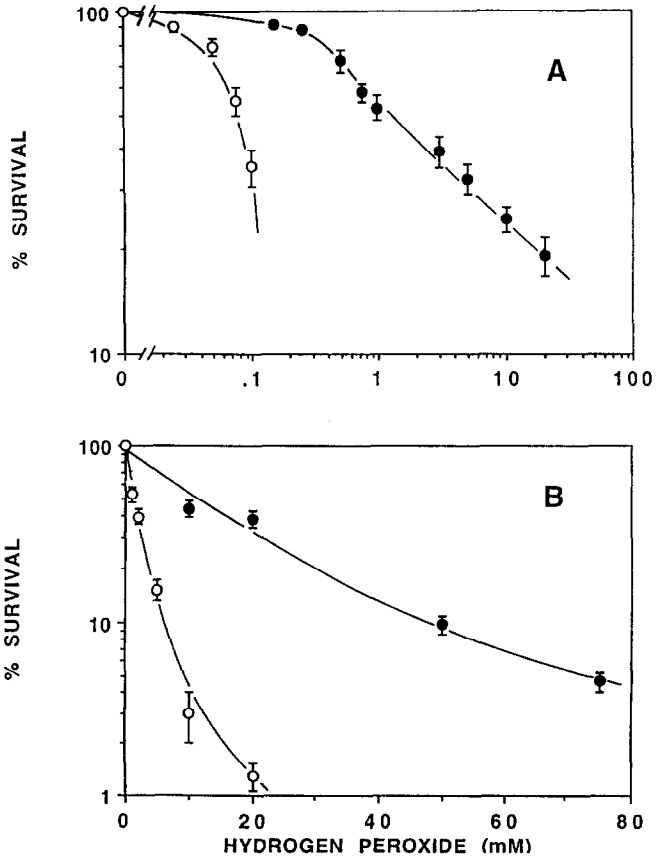

FIG. 1. Survival curves for AA8 and AG8 cells treated with H_2O_2. WT AA8 (○) and H_2O_2-resistant AG8 (●) cells were exposed for 30 min to increasing concentrations of H_2O_2 at either 37° (panel A) or 4° (panel B). Results are the means ± SEM of 3–4 separate experiments, each performed in duplicate.

TABLE 3. Cross-resistance of AG8 cells to other cytotoxic agents

Agent	AA8 IC_{50}*	AG8 IC_{50}*
H_2O_2†	78 ± 2 μM	1326 ± 54 μM‡
H_2O_2§	1.17 ± 0.11 mM	12.7 ± 1.3 mM‡
$CdCl_2$‖	0.33 ± 0.02 μM	1.33 ± 0.08 μM‡
$NaAsO_2$‖	19.5 ± 2.1 μM	44.5 ± 3.3 μM‡
TBHP†	300 ± 18 μM	878 ± 42 μM‡
CHP†	100 ± 8 μM	295 ± 10 μM‡
Menadione†	17.0 ± 0.9 μM	29 ± 1.5 μM‡
$HgCl_2$‖	50.0 ± 4.5 μM	76.0 ± 5.2 μM‡
Heat (43°)	26.0 ± 1.9 min	23.0 ± 1.6 min
254 nm UV light	8.0 ± 0.5 J/m^2	9.0 ± 0.4 J/m^2
γ-Rays	4.5 ± 0.2 Gy	4.6 ± 0.2 Gy
neutrons	1.8 ± 0.1 Gy	1.8 ± 0.1 Gy

* IC_{50} is defined as the concentration of a drug, dose of a physical agent, or duration of a treatment, that decreased the survival of AA8 or AG8 cells to 50% of that observed in control cells. The IC_{50} values were obtained from clonogenic survival experiments similar to those described in Fig. 1. Results are the means ± SEM of 3–4 separate experiments, each performed in duplicate.

† Thirty-minute exposure at 37°.

‡ Value for AG8 cells was significantly greater ($P < 0.05$) than for AA8 cells.

§ Thirty-minute exposure at 4°.

‖ Continuous exposure.

Cross-resistance Studies

The cytotoxic effects of several other DNA-damaging agents toward AA8 and AG8 cells were examined to determine whether the acquisition of the H_2O_2-resistant phenotype was accompanied by cross-resistance to other agents. As shown in Table 3, AG8 cells exhibited significant ($P < 0.05$) cross-resistance to $CdCl_2$ (~4-fold), $NaAsO_2$ (~2.3-fold), TBHP (~2.9-fold), CHP (~3-fold), menadione (~1.7-fold) and $HgCl_2$ (~1.5-fold). AA8 and AG8 cells were not significantly ($P > 0.05$) cross-resistant to heat (43°), 254 nm UV light, ^{137}Cs γ-radiation, and 42-MeV ($p \rightarrow Be^+$) fast neutrons (Table 3).

Sensitivity to H_2O_2-Mediated Depletion of Adenine Nucleotides

AG8 cells contained significantly ($P < 0.05$) more ATP and NAD^+ than WT AA8 cells when the data were expressed per 10^6 cells, whereas these levels were not significantly different ($P > 0.05$) when expressed per milligram of protein (Table 1). Treatment with increasing concentrations of H_2O_2 for 30 min at 37° lowered adenine nucleotide levels more efficiently in AA8 cells than in AG8 cells (Fig. 2). For example, 150 μM H_2O_2 decreased NAD^+ levels by ~50% in the WT cells, whereas ~1 mM H_2O_2 was necessary to produce a similar effect in the resistant cells. Similarly, 300 μM H_2O_2 decreased ATP levels by ~50% in WT cells, whereas ~3 mM H_2O_2 was necessary to cause a similar effect in AG8 cells.

Role of Catalase

Catalase activity in cell-free extracts of AA8 and AG8 cells was assessed by both spectrophotometric (H_2O_2 depletion)

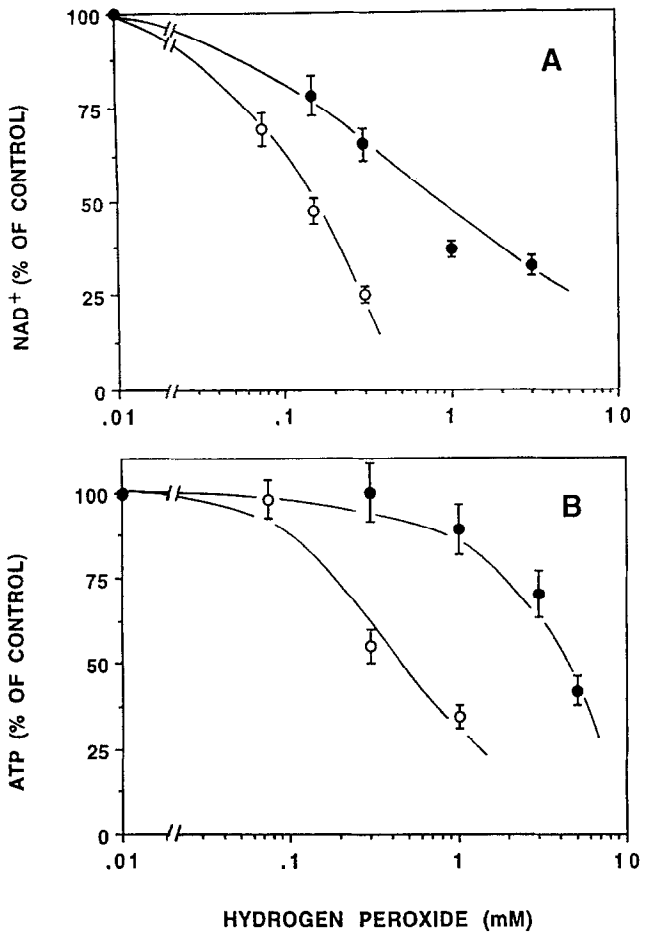

FIG. 2. H_2O_2-mediated depletion of NAD^+ and ATP in AA8 and AG8 cells. WT AA8 (○) and H_2O_2-resistant AG8 (●) cells were treated with increasing concentrations of H_2O_2 for 30 min at 37°. Their NAD^+ (panel A) and ATP (panel B) levels were then assayed and expressed on a per milligram of protein basis, as detailed in Materials and Methods. Initial (control) levels of NAD^+ were 6.5 ± 0.5 (AA8) and 7.0 ± 0.6 (AG8) nmol/mg of protein; initial (control) levels of ATP were 24.0 ± 1.9 (AA8) and 23.0 ± 1.9 (AG8) nmol/mg of protein (Table 1). Results are the means ± SEM of 3–4 independent experiments.

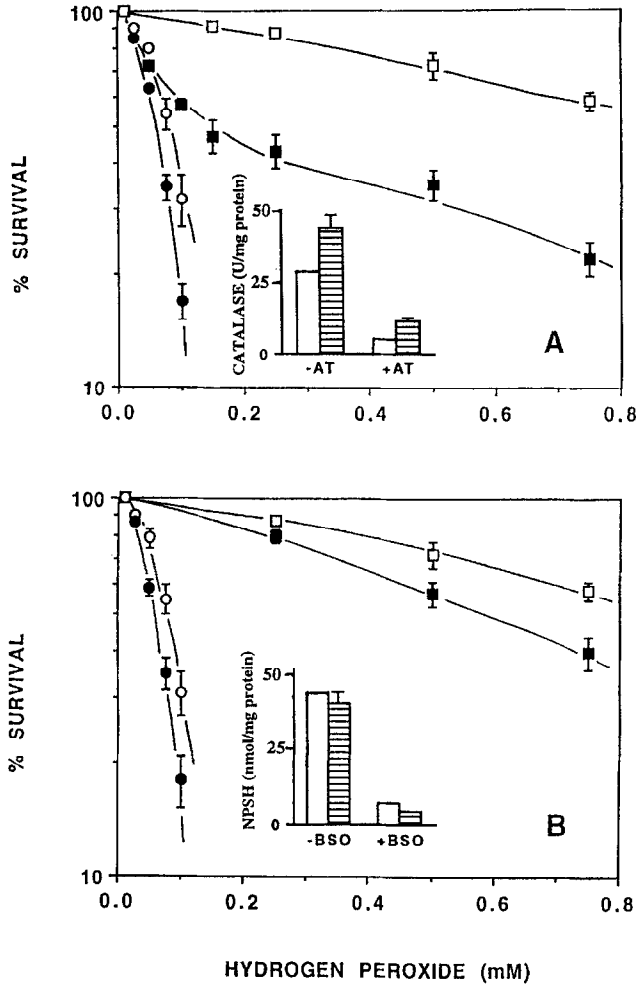

FIG. 3. Effect of depletion of catalase or NPSH on the sensitivity of AA8 and AG8 cells to H_2O_2-mediated cytotoxicity. (A) AA8 (circles) or AG8 (squares) cells were incubated for 6 hr at 37° in the absence (open symbols) or presence (closed symbols) of 10 mM AT and then were exposed to H_2O_2 at 37° for 30 min. Catalase activity in AA8 (open columns) and AG8 (striped columns) cells at the time of H_2O_2 treatment (as measured using the spectrophotometric assay) is shown in the inset. Data represent the means of 3–4 independent determinations; SEM was ≤10%. (B) AA8 (circles) or AG8 (squares) cells were incubated for 16 hr at 37° in the absence (open symbols) or presence (closed symbols) of 7.5 μM BSO and then were exposed to H_2O_2 at 37° for 30 min. NPSH levels in AA8 (open columns) and AG8 (striped columns) cells at the time of H_2O_2 treatment are shown in the inset. Data are the means ± SEM of 3–4 independent determinations.

and polarographic (oxygen evolution) methods. Although other differences between the cell-free extracts obtained from the two cell lines could impact on these assays, only differences in catalase activity *per se* should give the same result in both assays. The data in Table 1 indicate that the two methods gave similar results; H_2O_2-resistant AG8 cells displayed significantly ($P < 0.05$) greater catalase activity than WT AA8 cells irrespective of whether this was expressed per 10^6 cells (~3.5-fold increase) or per milligram of protein (~1.6-fold increase). Treatment for 6 hr with 10 mM AT decreased catalase activity to ~25% of control levels in both AA8 and AG8 cells (inset to Fig. 3A); a similar extent of catalase depletion was observed in Chinese hamster fibroblasts [7]. The sensitivity to H_2O_2 (30-min treatment at 37°) in the clonogenic survival assay under conditions of normal or decreased catalase activity was different in the two cell types. As illustrated in Fig. 3A, AT-treated AA8 cells were slightly but uniformly sensitized at all concentrations of H_2O_2. In contrast, AT-treated AG8 cells were markedly sensitized at low H_2O_2 concentrations; however, above ~200 μM H_2O_2 the survival curves for control and AT-treated AG8 cells ran parallel to each other, indicating that little further sensitization occurred.

Role of Cellular NPSH, GSH Reductase and GSH Peroxidase

The resistant AG8 cell line contained significantly ($P < 0.05$) more NPSH than WT AA8 cells when the data were

expressed per 10^6 cells, whereas NPSH levels expressed per milligram of protein were similar (Table 1). A 16-hr exposure of monolayer cultures to 7.5 μM BSO produced a large decrease in the intracellular NPSH content in both cell types (inset to Fig. 3B). Depletion of NPSH caused a slight (~1.5-fold) increase in H_2O_2-mediated cytotoxicity (30-min treatment at 37°) toward both AA8 and AG8 cells (Fig. 3B). However, the resistance of AG8 cells to H_2O_2 was clearly not reversed under these experimental conditions even though the NPSH levels in BSO-treated AG8 cells were *much* lower than in undepleted control AA8 cells.

AG8 cells exhibited significantly ($P < 0.05$) greater GSH reductase activity than WT cells regardless of whether the data were expressed per 10^6 cells (~2.9-fold) or per milligram protein (~1.3-fold) (Table 1). Several attempts were made to assess the activity of GSH peroxidase in AA8 and AG8 cells. Although a low activity of the enzyme was apparent in both cell lines, it was not possible to generate a linear response curve by adding progressively increasing amounts of cell extract. The assay, however, gave reliable results using lysates from both McCoy's cells and human erythrocytes. Thus, the level of GSH peroxidase appeared to be minimal in both AA8 and AG8 cells.

Sensitivity to H_2O_2-Mediated DNA SSB and DSB Induction

Treating AA8 or AG8 cells for 30 min at 37° with up to 50 μM H_2O_2 resulted in a concentration-dependent generation of SSBs (Fig. 4A). The yield of SSBs was slightly (~1.5 fold) although significantly ($P < 0.05$) decreased in the resistant AG8 cell line. To investigate the repair of the SSBs generated by H_2O_2 in the two cell lines, initial damage was induced by treatment for 10 min with 37.5 μM (AA8) or 50 μM (AG8) H_2O_2 (which induced similar levels of SSBs) followed by incubation at 37° in fresh medium for various times. The two cell lines repaired these SSBs with similar kinetics, with $T_{1/2}$ values of ~15 min in each case (data not shown).

Treating AA8 cells at 37° with survival-curve range concentrations of H_2O_2 (i.e. up to 200 μM) did not produce measurable levels of DSBs (Fig. 4B). Similarly, treating AG8 cells with up to 5 mM H_2O_2 (which decreased survival to ~30%) produced no measurable DSBs (Fig. 4B).

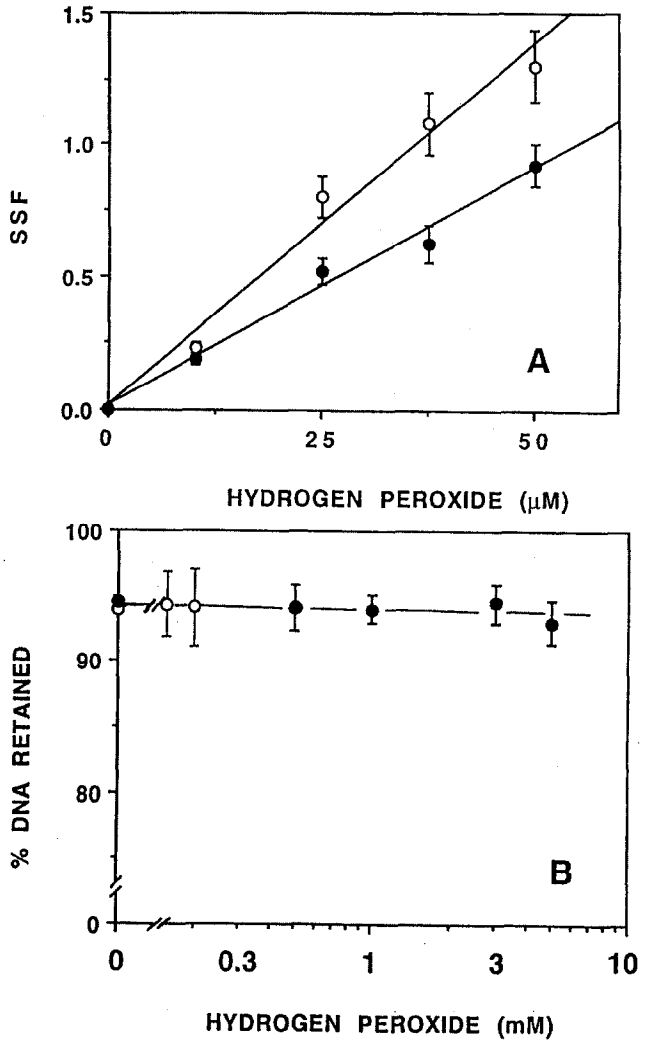

FIG. 4. H_2O_2-induced DNA SSBs and DSBs in AA8 and AG8 cells. WT AA8 (○) and H_2O_2-resistant AG8 (●) cells were exposed for 30 min at 37° to increasing concentrations of H_2O_2. DNA SSBs (panel A) or DSBs (panel B) were assessed by alkaline elution and pulsed-field gel electrophoresis, respectively. Results are the means ± SEM of 3–4 independent experiments, each performed in duplicate.

DISCUSSION

We here describe the development and preliminary phenotypic characterization of a novel cloned cell line (AG8) that was isolated from the WT CHO AA8 cell line (through the intermediacy of the V850 variant) on the basis of its resistance to H_2O_2. AG8 cells were highly resistant to the cytotoxic effects of H_2O_2, although the degree of resistance (~17-fold at 37°, ~11-fold at 4°) depended somewhat on the temperature of treatment with the oxidant. They were moderately (~3-fold) cross-resistant to two organic hydroperoxides, TBHP and CHP. AG8 cells were also moderately cross-resistant to $CdCl_2$ (~4-fold), $NaAsO_2$ (~2.3-fold), and to a lesser extent to $HgCl_2$ (~1.5-fold) and menadione (~1.7-fold), but were not significantly cross-resistant to several other agents known to exert their biological effects at the DNA-damage level, namely γ-rays, neutrons, and UV light. In the case of γ-rays and neutrons, cytotoxicity is believed to be mediated by DSBs [20], whereas H_2O_2 did not induce detectable levels of DSBs in either cell line over the range of oxidant concentrations where cell killing was observed (Fig. 4B). Thus, DSBs do not appear to mediate cell killing by H_2O_2, and these lesions are therefore not informative with respect to the mechanism by which AG8 cells have acquired resistance to H_2O_2. The lack of cross-resistance to UV light, a quintessential DNA-targeted agent, suggests that there is no difference in the DNA excision-repair capability of AA8 and AG8 cells. Although many of the lesions induced by H_2O_2

are likely to be repaired by the base excision-repair pathway (whereas UV light lesions are repaired by nucleotide excision), the lack of a DNA-repair component in the AG8 phenotype is supported by the similar rates of rejoining of H_2O_2-induced SSBs in the two cell lines.

These data suggest that the H_2O_2-resistant phenotype of AG8 cells has at least two components, the first of which reflects a general ~3-fold resistance to peroxides and heavy metals, and the second of which is specific to H_2O_2 and accounts for the remainder of their total ~17-fold resistance to this agent. Considering that the AG8 phenotype was developed following a single 1-hr exposure of the V850 variant to a high (1.5 mM) concentration of H_2O_2, and further considering that the V850 variant was itself developed by an adaptation approach where the cell culture was exposed to escalating concentrations of the oxidant, it appears that AG8 cells essentially represent a combination of two different phenotypes. The first component is presumably "inherited" from the V850 variant, does not involve catalase [8], and is responsible for the cross-resistance to the various additional ROI-inducing agents tested. This interpretation is supported by the observation that V850 cells exhibited similar degrees of resistance to $CdCl_2$ (~2.5-fold), $NaAsO_2$ (~3.6-fold), and $HgCl_2$ (~1.6-fold) [21] as did AG8 cells (Table 3). The second component, which is acquired following the single cytotoxic exposure to H_2O_2, appears to be H_2O_2-specific and is most likely mediated by an increased level of catalase, analogous to the H_2O_2-resistant Chinese hamster cells derived by Spitz and colleagues [7]. The increased catalase activity of AG8 cf. AA8 cells (Table 1), although modest (~1.6-fold based on protein content; ~3.6-fold per cell) when compared with the much greater increases observed in other H_2O_2-resistant cell lines (e.g. [7]), nonetheless probably accounts for most of the second, H_2O_2-specific, component of resistance, especially considering that the "intermediate" V850 variant cells actually displayed less catalase activity than AA8 cells [8].

To further investigate the role of catalase in the AG8 phenotype, we examined the effect of AT on the sensitivity of the two cell lines to H_2O_2. AT is a relatively specific inhibitor of catalase in Chinese hamster cells at non-toxic concentrations and has little effect on the activity of other antioxidant enzymes such as GSH transferase, GSH peroxidase, and superoxide dismutase (EC 1.15.1.1) [7]. AT markedly sensitized both AA8 and AG8 cells to H_2O_2, although this was accompanied by quite different effects on the shapes of the respective survival curves (Fig. 3A). Although AT-treated AG8 cells had significantly lower catalase levels than untreated AA8 cells, the former cells were nonetheless generally more resistant to H_2O_2. Thus, while catalase does have an important role in protecting both AA8 and AG8 cells against the cytotoxic effects of H_2O_2, these data confirm that elevated catalase activity is only a partial factor in the overall AG8 phenotype.

As a further indirect measure of catalase involvement, we compared the susceptibility of AA8 and AG8 cells to H_2O_2-mediated induction of DNA SSBs, lesions that are induced efficiently by H_2O_2 in mammalian cells [19, 22–29]. Although total SSBs are not always predictive of H_2O_2-mediated cytotoxicity (e.g. [30]), these lesions do serve as a dosimeter for the interaction between H_2O_2-derived ROIs and cellular DNA, and thus allow an estimation of the combined role of anti-oxidant mechanisms such as elevated catalase and GSH reductase. Despite their greatly different sensitivity to the cytotoxic effects of H_2O_2 (Fig. 1A), AG8 cells were only slightly (~1.5-fold) less susceptible than WT cells to SSB induction by the oxidant (Fig. 4A). It should be noted that, because of the sensitivity of the technique used, SSBs were measured after concentrations of the oxidant that produced very little cell killing, and these data may not extrapolate quantitatively to survival-curve range concentrations of H_2O_2. It is also important to note that, in these experiments, the H_2O_2 treatments were for 30 min at 37°, so the net level of SSBs detected will depend both on the level of breaks produced and on the fraction of these breaks that were removed during the treatment [25]. Since the rate of SSB removal was similar in the two cell lines, these differences in the SSB levels at 30 min presumably reflect a decreased susceptibility of AG8 cells to the formation of SSBs. These differences, although modest, do suggest that the observed increases in catalase and GSH reductase activity in AG8 cells may be responsible for the second component of their resistance to H_2O_2.

A further difference between the WT AA8 and H_2O_2-resistant AG8 cells was their susceptibility to adenine nucleotide depletion following treatment with H_2O_2. Such decreases in cellular NAD^+ levels following exposure to ROIs are believed to be caused by increased poly(ADP-ribose)polymerase (EC 2.4.2.30) activity triggered by the production of DNA strand breaks [25, 27, 28]. The decrease in cellular ATP levels may or may not be a consequence of this drop in NAD^+ levels. AG8 cells, although containing basal levels of ATP and NAD^+ similar to those detected in AA8 cells (per mg of protein; Table 1), displayed resistance to H_2O_2-induced depletion of these nucleotides (Fig. 2). These results, however, should be considered relative to the sensitivity of the two cell lines to H_2O_2-mediated cell injury (Fig. 1A), when it becomes apparent that AG8 cells actually undergo more extensive depletion of their cellular ATP and NAD^+ pools than WT cells when compared at equal survival levels. These results are difficult to rationalize with the assumption that H_2O_2-mediated strand breaks trigger NAD^+ depletion. The level of induced SSBs was only ~1.5-fold different in the two cell lines (Fig. 4A), and yet the level of NAD^+ depletion was almost 10-fold different. However, it should be noted that the NAD^+ depletion data were determined on cells exposed to survival-curve range concentrations of H_2O_2, whereas the levels of SSBs were measured after sublethal concentrations of the oxidant (see above).

Another potentially important modulator of ROIs in mammalian cells is NPSH. However, AA8 and AG8 cells contained similar NPSH levels (when the data were normalized to cellular protein content; Table 1) and depletion of intracellular NPSH caused only a slight (~1.5-fold) in-

crease in H_2O_2-mediated cytotoxicity in both cell lines (Fig. 3B). Thus, the resistance of AG8 cells cannot be explained on the basis of differences in their overall NPSH content. This conclusion is supported by the observation that the levels of GSH peroxidase, rather than GSH, are rate-limiting for the peroxidase-catalyzed inactivation of H_2O_2 [31]. Surprisingly, the GSH peroxidase activity of both AA8 and AG8 cells was extremely low compared with other cell lines (see above), so this enzyme is unlikely to be an important factor in the response of either cell line to H_2O_2 (and thus in the H_2O_2-resistant phenotype).

Finally, it is interesting to note that the increased size and protein content (~2.3-fold, Table 1) of AG8 cells compared with AA8 cells was associated with an insignificantly small (~1.1-fold; $P > 0.05$) increase in overall DNA content, indicating that the increase in protein content is not coupled to ploidy changes. It should also be noted that, in the studies of Spitz and colleagues [7], there was a relationship between ploidy and the stable H_2O_2-resistant phenotype.

In conclusion, the AG8 phenotype is particularly interesting in that it suggests that the two general mechanisms of resistance to H_2O_2 that have been identified previously in cultured Chinese hamster cells can occur within the same cell type *if* the appropriate environmental conditions for inducing the resistant phenotype are employed. The data suggest that the overall ~17-fold resistance of AG8 cells to H_2O_2 represents comparable contributions from: (i) an adaptive component that may or may not involve an increase in cellular antioxidant capacity but which is predictively related to the increase in overall cellular protein content, analogous to the variant lines described by Cantoni and colleagues [8, 9]; and (ii) a second component that does involve an increase in cellular antioxidant capacity, analogous to the cell lines described by Spitz and colleagues [7].

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