Differential Oxygen Radical Susceptibility of Adriamycin-sensitive and -resistant MCF-7 Human Breast Tumor Cells

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ABSTRACT

Recent evidence supports the concept that Adriamycin cytotoxicity may be mediated by drug semiquinone free radical and oxyradical generation. We tested this hypothesis further by exposing drug-sensitive (WT) and 500-fold Adriamycin-resistant MCF-7 human breast tumor cells (ADR®) to exogenous superoxide- and hydrogen peroxide-generating systems and subsequently monitored cell proliferation as a measure of cytotoxicity. The ADR® tumor cells tolerated a 4-fold greater exposure than sensitive cells to superoxide generated by the xanthine/xanthine oxidase system. Likewise, exposure to hydrogen peroxide produced by the action of glucose oxidase on glucose revealed a 4-fold diminished susceptibility of the drug-resistant cells to this reduced form of oxygen. Similar results were obtained by the direct application of hydrogen peroxide to cells. For both cell lines, cytotoxicity was dependent upon the magnitude and the duration of reactive oxygen exposure. When WT and ADR® cells were cultured under hypoxia (95% O2:5% CO2), in order to stimulate the intracellular production of oxyradicals, proliferation was inhibited to a greater extent in the drug-sensitive cell line. Additionally, hyperoxia potentiated the cytotoxicity of Adriamycin to both sensitive and drug-resistant cells, but the effect depended upon the concentration of the drug. Under hyperoxic conditions, Adriamycin caused oxygen radical-dependent cytotoxicity to the WT tumor cells at clinically relevant drug concentrations as low as 2 to 3 nM. With ADR® tumor cells, hyperoxia increased the cytotoxicity of Adriamycin at concentrations above 5 nM. Paradoxically, both the WT and the ADR® tumor cells were equally susceptible to the cytotoxic effects of γ irradiation. It is known that the Adriamycin-resistant MCF-7 cells greatly overexpress glutathione peroxidase and glutathione transferase activities; however, other biochemical defenses against reactive drug intermediates and oxygen radicals have been reported to be similar in the two cell lines. We have reexamined those observations in this report. The resistance of ADR® breast tumor cells to Adriamycin appears to be associated with a developmental tolerance to superoxide, most likely because of a twofold increase in superoxide dismutase activity, and a decreased susceptibility to hydrogen peroxide, most likely because of 12-fold augmented selenium-dependent glutathione peroxidase activity. Acting in concert, these two enzymes would decrease the formation of hydroxyl radical from reduced oxygen species. To this end, we have compared the susceptibility of Adriamycin to different biochemical and pharmacological characteristics of drug-resistant tumors compared to their drug-sensitive counterparts. Among these, the most conspicuous appear to be (a) a decreased net cellular accumulation of anticancer drugs, resulting from an efflux pump function of overexpressed membrane P-glycoproteins (4), (b) the elevation of reduced glutathione (5, 6), (c) diminished drug metabolic activation resulting from the down-regulation of monooxygenase and other enzyme activities (7), and (d) increased levels of drug- and reactive oxygen-detoxifying enzymes such as glutathione S-aryltransferases and selenium-dependent glutathione peroxidase (8, 9). Currently, it is not known if any one of these postulated mechanisms predominates in the resistance of tumor cells to Adriamycin or other anticancer agents, and it is conceivable that each of them contributes to some degree to the development of resistance.

Although still controversial, the mechanism of Adriamycin cytotoxicity, at least in some tumors, appears to be linked to its enzymatic reductive activation to a semiquinone free radical metabolite with subsequent generation of extremely toxic reactive oxygen species (10, 11). These activated forms of oxygen: superoxide anion radical, hydrogen peroxide and ultimately hydroxyl free radical, have been implicated in the cytotoxicity of Adriamycin in several in vitro tumor model systems (12-15), including MCF-7 human breast tumor cells (14, 15). Recently, an acquired subline ADR® was derived from the MCF-7 parental wild-type tumor cell line by slowly escalating Adriamycin exposure (8). This resistant subline is 500-fold resistant to Adriamycin, shows diminished ability to generate hydroxyl radical in the presence of Adriamycin (15, 16), exhibits three- to 9-fold decreased anthracycline accumulation depending upon the drug concentration, and has concurrently developed the phenotype of multidrug resistance, to include resistance to VP-16, actinomycin D, and vincristine (8, 9). In addition, the ADR® subline shows enhanced expression of DNA sequences homologous to the P-glycoprotein gene (17), enhanced glycolysis and ATP production (18), and stimulated pentose-shunt activity in the presence of exogenous peroxides (19).

We have designed the present study in order to examine the possibility that the diminished cytotoxicity of Adriamycin to ADR® drug-resistant tumor cells may be related to their ability to tolerate exposure to Adriamycin-mediated reactive oxygen species. To this end, we have compared the susceptibility of Adriamycin-sensitive (WT) and Adriamycin-resistant MCF-7 breast tumor cells (ADR®) to several reactive oxygen systems. Additionally, we have investigated the cytotoxic interaction of Adriamycin and hyperoxia to both sensitive and resistant tumor cells.
cells. It appears that higher levels of enzymatic defenses against toxic reactive oxygen species in the resistant tumor cells provide significant protection against oxyradical exposure. This finding suggests that elevated levels of oxygen free radical defenses, likewise, can at least partially modulate the cytotoxicity of Adriamycin in the multidrug-resistant MCF-7 human breast tumor model.

MATERIALS AND METHODS

Chemicals and Drugs. Xanthine, xanthine oxidase (50 units/ml), superoxide dismutase (3050 units/mg protein), catalase (10,000 units/mg protein), glucose oxidase (242 units/mg protein), NADPH, reduced glutathione, glutathione reductase (500 units/mg protein), bovine serum albumin, EDTA, cytochrome c, diethylenetriamine-pentaacetic acid, 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide, and 5,5'-dithiobis-nitrobenzoic acid were from Sigma Chemical Co. (St. Louis, MO); hydrogen peroxide and sulfosalicylic acid were from Fisher Chemical Co. (Fair Lawn, NJ). Desferal mesylate was from BenVenue Labs. (Bedford, OH), ultra-pure Tris was purchased from Bethesda Research Labs. (Gaithersburg, MD), and all other chemicals were of the highest purity available. Adriamycin hydrochloride (NSC 123127) was provided by the Drug Development Branch, National Cancer Institute, NIH, Bethesda, MD). The water used in this study was first deionized then distilled in glass.

Tumor Cell Culture and Cytotoxicity Assays. MCF-7 human breast tumor cells (WT) were grown in improved modified essential medium, supplemented with 2 mM L-glutamine, 2 mg/liter L-proline, 50 µg/ml gentamicin and 5% fetal bovine serum (Grand Island Biological Co., New York), at 37°C in a humidified incubator under an atmosphere of 5% carbon dioxide in air. The Adriamycin-resistant MCF-7 tumor cells, were derived from the parental line by stepwise selection in increasing concentrations of Adriamycin until the cells were capable of propagating in 10 µM drug, as described previously (8). Typically, the resistant cells were grown for a minimum of four passages in medium lacking Adriamycin before they were used in experiments. Tumor cell cytotoxicity assays were conducted by seeding 50,000 cells/well in 6-chambered Linbro dishes (Costar) in complete medium, then 24 to 30 h later, fresh medium containing the appropriate concentrations of Adriamycin, oxyradical-generating system, or other agents was added, the cells were incubated for various times (minutes to 24 h-see figure legends), replplenished with fresh medium, and grown for 72 h longer under normal conditions before the cells were trypsinized and counted with a model ZM Coulter counter (Hialeah, FL). In the experiments with hydrogen peroxide, this agent was diluted in glass-distilled water, then added directly to the cells in wells containing freshly replaced complete medium; an equal volume of water was added to the controls. In some experiments, the clonogenic assay was used to estimate cytotoxicity by inoculating 300–500 cells/well after exposure to the toxins, growing the cells for 12 days and counting the resulting colonies after washing with ethanol and staining with methylene blue. Cytotoxicity was usually determined by using a range of concentrations of drug or toxic oxygen species and inhibitor concentration 50% (IC50) values were derived from computer plots (Macintosh).

Superoxide- and Hydrogen Peroxide-Generating Systems. To study the relative susceptibility of the WT and ADR® breast tumor cells to an extracellular exposure of reactive oxygen species, two enzymatic oxyradical generating systems were used. The first was the xanthine/xanthine oxidase reaction that produces superoxide and hydrogen peroxide during the enzymatic conversion of xanthine to urate. Tumor cells growing in monolayers were exposed to various activities of this enzyme in the presence of 1 mM xanthine in complete medium for a fixed time of 60 min, or to 10 µM of xanthine oxidase and 1 mM xanthine for various times. The reaction mixtures were then aspirated, fresh medium was added and the inhibition of cell growth was assessed 3 days later. The second enzymatic system, the glucose oxidase-mediated oxidation of glucose to gluconic acid, reduces molecular oxygen directly to hydrogen peroxide. We investigated the effects of glucose oxidase activities and the duration of exposure to this hydrogen peroxide-generating system on the survival of sensitive and drug-resistant tumors cells. The concentration of glucose was initially 11 mM in all experiments. These two enzymatic oxyradical-generating systems provided the capability to carefully control both the rate and the amount of superoxide and hydrogen peroxide exposed to the cells. In some experiments, we also studied the toxicity of hydrogen peroxide directly added to the medium.

Exposure of the Tumor Cells to Hyperoxic Conditions. Hyperoxia (>21% oxygen) can cause leakage of reactive oxygen species from oxidoreductase enzymes such as NADPH-cytochrome P-450 reductase and mitochondrial respiratory electron transport chain enzymes (20–23) and from the autodestruction of reduced cellular biochemistry. WT and ADR® cells were exposed to an atmosphere of 95% oxygen-5% carbon dioxide using a Forma CH/P incubator with oxygen control for various lengths of time (1 to 4 days) and the effects of that >4-fold increased oxygen level on cell proliferation were examined. In these experiments the cells were inoculated into 6-well plates and allowed to attach and begin to proliferate for 24 to 30 h prior to initiating the exposure to 95% oxygen. Adriamycin was included in the medium during the hyperoxic exposure in separate experiments.

Biochemical and Enzymatic Assays. Prior to determining the activities of oxidoreductase enzymes known to activate Adriamycin and enzymes known to play important roles in oxyradical detoxification, the WT and ADR® tumor cells were trypsinized and washed several times in ice-cold phosphate buffered saline, or scraped off the monolayers directly into the appropriate chilled buffers, centrifuged and resuspended in buffer prior to sonication with a microprobe at setting 5, for three 10-s bursts (model W-225; Heat Systems Ultrasonics, Inc., New York). Cytosolic enzymes were then assayed in the supernatants following centrifugation in an Eppendorf microfuge for 20 min. For some measurements, microsomal (24) and mitochondrial (25) subcellular fractions were isolated by differential centrifugation techniques. NADPH-cytochrome P-450 reductase activities were assayed in the microsomal fractions from tumor cells using cytochrome c as the electron acceptor (26), and NADH-dehydrogenase activities were estimated by monitoring the reduction of cytochrome c by tumor mitochondria in the presence of NADH. Glutathione peroxidase activity was determined by the method of Paglia and Valentine (27) with slight modifications, using both H2O2 and cumene hydroperoxide, glutathione S-aryl transferase activity was assayed by the reaction of reduced glutathione (1 mM) with chlorodinitrobenzoate (28), and reduced glutathione levels were measured in tumor cell sonicates deproteinated with sulfosalicylic acid by the method of Ellman (29). Superoxide dismutase activities were assayed in tumor cell sonicates by the method of McCord and Fridovich (30), using the acetylcysteine c modification as described by Azzi et al. (31). Protein determinations were by the method of Lowry et al. (32).

γ Irradiation of Cells. To determine whether there were differences in the relative toxic effects of intracellular oxygen radicals generated by irradiation, WT and ADR® tumor cells were trypsinized, suspended in chilled complete medium, and exposed to various doses of γ radiation with a Shepherd Mark I model 68 137Cs irradiator with a dose rate of 5.74 Gy/min. Tumor cells were then inoculated in triplicate at 50,000 cells/well for proliferation inhibition assays and at 500 cells/well for the clonogenic assay of cytotoxicity.

Statistics. Data were analyzed by Student’s t test (33), and differences between mean values at P < 0.05 were considered to be significant.

RESULTS

Adriamycin Cytotoxicity to MCF-7 WT and ADR® Tumor Cells. It is known that there are marked differences in the ability of Adriamycin to inhibit the proliferation of sensitive and drug-resistant MCF-7 breast tumor cells (8, 9, 16). As shown in Fig. 1, the ADR® subline was found to be more than 500-fold resistant to Adriamycin than the parental WT cells. The IC50 values for Adriamycin by continuous exposure were 5 to 7 nM and 2 to 3 μM for the WT and ADR® cells, respectively. The ADR®-resistant phenotype has been previously reported to be
about 200-fold resistant to Adriamycin, and to maintain stable resistance over extended subculture for at least 12 months (8). However, in the present study, although the IC_{50} value for Adriamycin toward the ADR^R cells was almost identical to that value published previously (8), the IC_{50} for the WT MCF-7 cells was considerably lower. This apparent change in susceptibility to Adriamycin may result from temporal alterations in the biochemistry of the WT cells or be due to slight differences in the methods for the determination of the IC_{50} values, such as the inoculation density or the number of drug concentrations used in the experiments. In the experiments conducted in this study, we used cells showing a relative 500-fold resistance to Adriamycin by the continuous drug exposure, inhibition of proliferation assay.

Sensitivity of WT and ADR^R Cells to Superoxide Free Radical.

The xanthine oxidase/xanthine system was used as a means to expose tumor cells to an external source of superoxide. This system also produces hydrogen peroxide and most likely, hydroxyl radical in the presence of trace quantities of transition metal ions found in the cell culture medium, but the initial and predominating enzymatically reduced form of oxygen is superoxide. When the WT and the ADR^R cells were exposed to the xanthine oxidase system, we observed a differential cytotoxicity of superoxide to the sensitive and drug-resistant cells. In the presence of 1 mM xanthine, the amounts of xanthine oxidase required to diminish cell numbers by 50% following a 60 min exposure were calculated to be 3 and 9 units/ml for the WT and the ADR^R cells, respectively (Fig. 2A). Incubating the cells with either xanthine oxidase alone at 100 units/ml or with xanthine alone up to 3 mM resulted in no substantial cytotoxicity (<5%) to either cell line (data not presented). In Fig. 2B, it is shown that the duration of exposure to the superoxide-generating system also resulted in differential cytotoxicity; 50% killing of the WT cells required an 8- to 10-min exposure, in contrast, 50% killing of the ADR^R cells required about 45 min. The xanthine oxidase reaction at 10 units/ml capable of generating 1.8 \mu mol of superoxide during the 60-min exposure period. Thus, the Adriamycin-resistant cells were comparatively three- to four-fold more resistant to superoxide exposure in the extracellular environment than the Adriamycin-sensitive cells.

Cytotoxicity to Hydrogen Peroxide by Glucose Oxidase.

As mentioned above, the xanthine oxidase system forms both superoxide anion and hydrogen peroxide as products of xanthine oxidation. There is considerable evidence that superoxide itself is neither reactive nor especially toxic, and that damage to cells or tissues exposed to superoxide results from the formation of the hydroxyl radical through the intermediary of hydrogen peroxide (34, 35). We therefore studied the inhibition of tumor cell proliferation by hydrogen peroxide generated by the glucose oxidase-catalyzed oxidation of glucose. As shown in Fig. 3A, the addition of various amounts of glucose oxidase to the cells revealed that the WT cells, compared to the ADR^R cells, had a substantially decreased ability to resist a peroxide-induced oxidant stress. The IC_{50} values for the sensitive and drug-resistant cells were found to be 4 and 16 units/ml of glucose oxidase, respectively, for a 60-min exposure. The time course for this system (Fig. 3B) also showed a significant difference between the two cell lines in susceptibility to hydrogen peroxide.

Direct Addition of Hydrogen Peroxide.

Hydrogen peroxide added as a bolus to tumor cells was also very toxic, and as with the xanthine oxidase and glucose oxidase systems, there was a divergence in the toxicity curves for the WT and ADR^R cells (Fig. 4). The IC_{50} values for hydrogen peroxide (60-min exposure) were estimated to be about 30 and 85 \mu M, respectively, for the WT and ADR^R breast tumor cells. In order to probe for the participation of hydroxyl radical in hydrogen peroxide cytotoxicity, we added metal ion chelators to the medium prior to the addition of the peroxide, with the expectation that chelators might block hydroxyl radical generation by removing metal ions (iron and copper) that could catalyze the Fenton reaction (36). Neither diethylenetriaminepentaacetic acid at 10 \mu M nor desferal as high as 1 mM had any sparing effect on the cytotoxicity of hydrogen peroxide to either cell line, although there was some delayed toxicity resulting from the exposure to desferal (data not presented). Because these two highly charged...
chelators do not rapidly penetrate cell membranes, this lack of protection against hydrogen peroxide cytotoxicity suggests that metal ion-catalyzed conversion of hydrogen peroxide to hydroxyl radical or other reactive metal-containing oxidant did not occur outside of the cells.

**Hyperoxia Toxicity.** When cells are exposed to high concentrations of isotonic oxygen, the respiratory electron transfer enzymes of the mitochondria and the flavin oxidoreductase enzymes of the endoplasmic reticulum divert electrons to molecular oxygen. This process greatly enhances the leakage of superoxide and hydrogen peroxide from these sources (20-23). Thus, hyperoxia exposure results in the intracellular production of several reactive oxygen species. The cytotoxic effects of exposure of MCF-7 WT and ADRR tumor cell lines to hyperoxia for several days is shown in Fig. 5, A and B. After a period of 24 h under 95% oxygen, there was only a marginal decrease in the number of WT and ADRR cells compared to euoxic controls, indicating that this exposure time was not sufficiently toxic to inhibit cell proliferation or to kill the cells and cause them to release from the monolayer. Longer exposure periods of up to 96 h revealed that hyperoxia was selectively more toxic to the sensitive cells than to the drug-resistant subline. It appeared that while WT cell proliferation was considerably slowed during long-term hyperoxia (Fig. 5A), the ADRR cells were less susceptible to hyperoxia (Fig. 5B). These results are consistent with the previous observations of differential susceptibility of these two cell lines to extracellular oxyradical exposure, as observed in the superoxide and hydrogen peroxide experiments described above.

**Potentiation of Adriamycin Cytotoxicity by Hyperoxia.** Because Adriamycin may be cytotoxic as the result of its ability to redox cycle and generate reduced oxygen species, we reasoned that the combination of hyperoxia and Adriamycin should result in additive or possibly synergistic toxicity. Indeed, when tumor cells were exposed to Adriamycin during hyperoxic (95% oxygen) conditions, the cytotoxicity of the drug relative to euoxic (21% oxygen) controls was enhanced. As shown in Fig. 6, hyperoxia potentiated the cytotoxicity of low concentrations of Adriamycin to the WT drug-sensitive cells, but in contrast, hyperoxia increased the cytotoxicity of Adriamycin to the ADRR cells only at the higher drug concentrations. These results appear to validate the hypothesis that oxyradicals are important to the mechanism of Adriamycin cytotoxicity to the MCF-7 WT cells. It is possible that oxygen radical generation may be relatively less important in the cytotoxicity of the drug to ADRR cells than WT cells.

**γ Irradiation.** There is considerable evidence that ionizing radiation causes cell damage in part by an oxyradical-mediated mechanism (37). When tumor cells were exposed to 137Cs as a source of γ radiation, survival of both WT and ADRR cells at 3 and 5 Gy was almost identical, as determined by both the inhibition of proliferation and by the clonogenic assays (Fig. 7). These results showing no resistance to irradiation by the ADRR cells contrast with the previously observed decreased cytotoxicity of superoxide and hydrogen peroxide to this subline. It may be that oxygen radicals generated within the nucleus of tumor cells are unable to be scavenged by cytosolic enzymatic oxyradical defenses that are known to be elevated in the resistant cell line (8, 16). An alternative explanation is that the
radiolysis of water produces hydroxyl radical directly, and this pathway is not linked to the sequential single electron reduction of molecular oxygen process that occurs in enzymatic systems.

Biological Parameters of Sensitive and Drug-Resistant MCF-7 Tumor Cells. In a previous publication it was reported that several biochemical parameters relevant to Adriamycin cytotoxicity were elevated in the ADR^r tumor subline compared to the drug-sensitive WT parental line (8, 16). Because the IC_{50} value for Adriamycin toward the WT tumor cells appeared to have decreased slightly from the previously reported value, and because of the possibility that extended culture of both sensitive and resistant cell lines might have resulted in altered biochemistry, we examined a number of enzymatic and biochemical variables that might influence either the activation of Adriamycin or the detoxification of reactive forms of oxygen. In addition, several of the enzymatic activities were previously measured in sonicates of tumor cells, and we decided to more closely examine select enzymes in purified subcellular fractions to determine if there were subtle differences between the sensitive and drug-resistant tumor cells. These biochemical and enzymatic measurements are summarized in Table 1. The activities of two enzymes known to activate Adriamycin, NADPH-cytochrome P-450 reductase and NADH-dehydrogenase, were roughly equivalent in microsomes and mitochondria isolated from the WT and ADR^r tumor cells. The slightly lower activity of cytochrome P-450 reductase in the resistant cells (65% of the sensitive cell value) would not be expected to result in dramatically diminished Adriamycin activation to its free radical intermediate. NADH-dehydrogenase activity was nearly two-fold higher in purified mitochondria from ADR^r tumor cells, ruling out any diminished ability to activate Adriamycin by this mitochondrial enzyme in the drug-resistant subline. The activity of superoxide dismutase was found to be almost two-fold higher in the ADR^r cells, suggesting the differential susceptibility of these cells to superoxide formed in the xanthine oxidase system might be attributed to the increased ability to scavenge superoxide by these tumor cells. The most interesting enzymatic differences between the WT and the ADR^r tumor cells were the marked increases in the activities of glutathione S-aryl transferase (14-fold) and glutathione peroxidase (7 to 12-fold). It has been previously reported that these two enzymes were greatly elevated in the ADR^r cells (8), but the absolute values were slightly different. Because the increased peroxidase activity of the ADR^r tumor cells was nearly equivalent with either hydrogen peroxide or cumene hydroperoxide as cosubstrate, it appears this activity is predominantly associated with the selenium-dependent glutathione-peroxidase. The increased activity of glutathione peroxidase would theoretically confer upon the resistant cells an increased capability to detoxify Adriamycin free radical-enhanced or glucose oxidase-generated hydrogen peroxide. We found the catalase activity to be similar in both cell types, as previously reported (16). Of note, is the measurement that the resistant cells had nearly threefold lower levels of reduced glutathione, the cofactor for the transferase and peroxidase enzymes. However, the concentration of glutathione in the resistant cells (6.1 ± 2.3 nmol/million cells, or about 3 mm) would still be sufficiently high to provide ample reduced glutathione to both glutathione transferase and glutathione peroxidase. No important differences were found in the protein content of the WT and ADR^r cells.

**Table 1 Comparison of several enzymatic and biochemical parameters in MCF-7 sensitive and Adriamycin-resistant breast tumor cells**

<table>
<thead>
<tr>
<th>Parameter^a</th>
<th>Sensitive tumors</th>
<th>Resistant tumors</th>
<th>R/S</th>
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</thead>
<tbody>
<tr>
<td>Microsomal NADPH-cytochrome P-450 reductase</td>
<td>66 ± 9^b</td>
<td>49 ± 10(^c)</td>
<td>0.74</td>
</tr>
<tr>
<td>Mitochondrial NADH-dehydrogenase</td>
<td>53 ± 2</td>
<td>96 ± 3(^c)</td>
<td>1.80</td>
</tr>
<tr>
<td>Glutathione peroxidase with hydrogen peroxide</td>
<td>0.74 ± 0.22</td>
<td>8.9 ± 1.1(^c)</td>
<td>12.0</td>
</tr>
<tr>
<td>Glutathione peroxidase with cumene hydroperoxide</td>
<td>1.71 ± 0.6</td>
<td>11.6 ± 3(^c)</td>
<td>6.8</td>
</tr>
<tr>
<td>Glutathione S-aryltransferase</td>
<td>10.4 ± 2.0</td>
<td>144 ± 45(^c)</td>
<td>14</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>3.0 ± 0.7</td>
<td>5.4 ± 1.0(^c)</td>
<td>1.80</td>
</tr>
<tr>
<td>Catalase</td>
<td>14.9 ± 3.8</td>
<td>10.2 ± 0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Reduced glutathione (nmol/10^6 cells)</td>
<td>16.5 ± 5</td>
<td>6.1 ± 2.2(^c)</td>
<td>0.38</td>
</tr>
<tr>
<td>Reduced glutathione (nmol/mg protein)</td>
<td>45 ± 11</td>
<td>19 ± 6.8</td>
<td>0.42</td>
</tr>
<tr>
<td>Protein (mg/10^6 cells)</td>
<td>366 ± 47</td>
<td>321 ± 27</td>
<td>0.88</td>
</tr>
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</table>

^a Enzyme activities are expressed as nmol of product formed/mg of protein/min, except superoxide dismutase activity is expressed units/mg protein where one unit is defined as the amount of enzyme necessary to inhibit by half the superoxide-dependent reduction of acetylcytochrome c. Catalase is expressed as \(\mu\)mol of hydrogen peroxide decomposed/mg protein/min and glutathione peroxidase activity is expressed as the rate of NADPH oxidation (mmol/mg protein/min) in the glutathione reductase-coupled assay using either hydrogen peroxide (0.073 mm) or cumene hydroperoxide (1.2 mm) as cosubstrates.

^b Data are expressed as the mean ± SD for three to 12 determinations.

^c Denotes a statistically significant difference when compared to WT values at \( P < 0.05 \).

**DISCUSSION**

The development of acquired multi-drug resistance by malignant cells is undoubtedly a major barrier to the effective chemotherapy of neoplastic diseases, and although there have been fragmentary advances in our understanding of the biochemistry and genetics of the resistance phenomenon, the known mechanisms of drug resistance have not provided clinically useful strategies for the reversal of this problem. While numerous in vitro models of drug resistance by tumor cells in culture have been developed, usually by the selective pressure of anticancer drug exposures, no consistent universal mechanism for resistance has emerged, and the relationships of the pharmacological, biochemical, and genetic characteristics of these tumor cell models to clinical multidrug resistance are only recently becoming known.

In the present study, we have hypothesized that the emergence of resistance to Adriamycin by MCF-7 breast tumor cells might be related to the metabolism of the drug to an activated semiquinone free radical intermediate and the subsequent production of reactive oxygen species that results from the redox cycling of the drug semiquinone (10, 11). Superoxide and hydroxyl free radicals and hydrogen peroxide are generated by this process, and these species avidly react with and damage a broad range of cellular constituents including membrane phospholipids, enzymes, and DNA (38). Oxygen free radicals are known to cause single-strand breaks in DNA (39) and to induce oxidative modifications in DNA bases (40). These molecular events have been postulated to account for the mutagenicity of oxyradicals to bacteria (41) and to mammalian cells (42). Furthermore, it has been clearly shown that Adriamycin can cleave DNA by an oxygen free radical-mediated process (11, 43, 44). We thus reasoned that if oxyradicals played a role in the development of resistance to Adriamycin, then tumor cells selected for resistance to Adriamycin should also demonstrate resistance to the direct cytotoxic effects of oxyradicals, thereby providing a test of the hypothesis.
The involvement of oxyradicals and oxidative stress in the mechanism of tumor cell killing by Adriamycin is controversial, but convincing, nascent evidence supporting this possibility is beginning to become available. For example, first Sato et al. (12) and later Doroshow et al. (13) have shown that Ehrlich ascites tumor cells and MCF-7 human breast tumor cells (14) generate increased amounts of reduced oxygen metabolites as the result of Adriamycin activation. Moreover, exogenous superoxide dismutase, catalase, and hydroxyl radical scavengers were shown to partially protect MCF-7 tumor cells from Adriamycin cytotoxicity (14), thus supporting the involvement of oxyradicals in the mechanism of cell kill. There is also some evidence that Adriamycin may be selectively toxic to well-oxygenated tumor cells (45), and another report indicated that differential cytotoxicity of daunomycin in tumor cells was related to their ability to detoxify hydrogen peroxide by a glutathione-dependent mechanism (46). Most recently, Sinha et al. (15) demonstrated by electron spin resonance spin-trapping techniques, that Adriamycin increased hydroxyl free radical production in drug-sensitive MCF-7 breast tumor cells, but in contrast, hydroyxl radical generation in 200-fold Adriamycin-resistant MCF-7 tumor cells was nearly absent. This technique specifically identifies hydroxyl free radical. In a separate report, Sinha et al. (16) demonstrated that a 5-fold differential hydroxyl radical formation in WT and ADRR tumor cells exposed to Adriamycin was not the result of diminished activation of Adriamycin by flavin monoxygenases, but could be attributed to enhanced activities of cytosolic reactive oxygen detoxifying enzymes in the resistant tumors. These investigators also reported partial protection against Adriamycin toxicity in both sensitive and resistant tumors by preincubating the cells with superoxide dismutase and catalase (16). Thus, it can be concluded, at least in MCF-7 breast tumor cells, that Adriamycin cytotoxicity can be linked to the metabolic overproduction of superoxide radical and hydrogen peroxide with conversion of these species to hydroxyl free radical, and possibly alkoxy radicals or metal-bound oxidants such as ferryl or perferryl iron species. We believe it is most likely hydroxyl radical that damages critical biomolecules because its reactivity is limited only by its diffusion rate.

The results presented in the present paper extend previous observations of oxyradical participation in tumor killing by Adriamycin into the area of oxyradical involvement in the development of resistance to this agent. Clearly, ADRR tumor cells selected for resistance to Adriamycin are significantly less susceptible than the WT Adriamycin-sensitive line to the toxic effects of exogenous superoxide and hydrogen peroxide generated by the xanthine oxidase/xanthine system. This differential susceptibility may be attributed to a twofold elevation in superoxide dismutase activity in the resistant cells, however, more likely, the difference in toxicity results from an increased detoxification of hydrogen peroxide, produced from the dismutation of superoxide free radical, by the 7- to 12-fold elevated glutathione peroxidase activity of the Adriamycin-resistant line. This interpretation is supported by the additional observation that Adriamycin-resistant cells are similarly resistant to extracellular hydrogen peroxide, as demonstrated by the experiments with the glucose oxidase system or by the direct addition of hydrogen peroxide to the tumor cells.

The effectiveness of glutathione peroxidase in detoxifying hydrogen peroxide by reducing it to water lies in its ability to scavenge extremely low concentrations of hydrogen peroxide, and further, glutathione peroxidase is capable of reducing secondary toxic aliphatic and aromatic peroxides to relatively inert alcohols (47). The peroxidase activity of the ADRR tumors is associated with the selenium-containing glutathione-peroxidase based on its ability to reduce both hydrogen peroxide and cumene hydroperoxide. Non-selenium glutathione peroxidase (glutathione transferase) cannot use hydrogen peroxide as a substrate. This observation with WT and ADRR tumor cells has also been reported by Kramer et al. (48). Recently, other investigators in our laboratory have used a cDNA probe specific for the human selenium-dependent peroxidase to show that ADRR tumor cells contain increased mRNA for this enzyme, revealing that the increased peroxidase activity of these cells is associated with the selenium-containing peroxidase.3

The comparative effectiveness of superoxide dismutase, catalase, and glutathione peroxidase in protecting cells from the toxic effects of oxyradicals was recently tested by quantitative microinjection of these enzymes into fibroblasts prior to exposing the fibroblasts to hyperoxia, and it was concluded that glutathione peroxidase offered much more protection from hyperoxia than the other two enzymes (49). In a variety of other test systems, the importance of glutathione peroxidase as a cellular defense against oxyradicals has been emphasized (50), thus we conclude that this enzyme is most likely responsible for the resistance toward oxyradicals by ADRR tumor cells.

While the xanthine oxidase and the glucose oxidase systems were used to expose attached tumor cells to extracellular reactive forms of oxygen, the hyperoxia experiments compared the susceptibility of the WT and the ADRR tumors to elevated intracellular generation of oxyradicals. In these experiments, the difference in oxyradical toxicity between the two cell types was not apparent until several days of exposure to the elevated oxygen concentrations. The divergence of the proliferation curves of the WT line under euvoxic and hyperoxic conditions may be the consequence of the inability of these cells to detoxify a constant increased rate of production of superoxide and hydrogen peroxide by electron transport enzymes. It is likely that the ADRR tumors were better able to withstand the elevated intracellular production of oxyradicals because of their higher levels of superoxide dismutase and glutathione peroxidase. At this time we do not know what biochemical targets are involved in hyperoxia toxicity to MCF-7 tumor cells or whether the targets for oxyradicals generated inside the cells are the same as those for oxyradicals formed outside the cells. The most conspicuous target for extracellular oxyradicals would be the cell membrane; intracellular oxyradicals could damage multiple critical biomolecules and could even attack and damage the cell membrane from inside.

The production of superoxide and hydrogen peroxide by both cell types under the hyperoxic conditions would be expected to be similar based upon the nearly equivalent activities of NADPH-cytochrome P-450 reductase and NADH-dehydrogenase. However, it is possible that secondary biochemical changes occurred in the MCF-7 tumor cells during several days growth under hyperoxic conditions. Among the first biochemical alterations in response to oxidative stress are the rapid loss of reduced glutathione and the inactivation of thiol-containing enzymes as a result of sulphydryl group oxidation (21, 51). After several days of hyperoxia, the levels of antioxidant defenses might have increased selectively in the resistant tumor cells. These possibilities are currently under examination in both the WT and ADRR cells.

Oxyradical production by Adriamycin redox cycling is dependent upon the availability of adequate oxygen concentra-

3 A. Townsend and K. Cowan, personal communication.
cations. Mimnaugh et al. (52) have shown that Adriamycin redox cycling leading to greatly enhanced lipid peroxidation in a microsomal system was very oxygen-dependent, with more than 5-fold greater lipid peroxidation at 100% than at 21% oxygen. There is also evidence in intact tissue for an oxygen-dependence of Adriamycin-caused toxicity, especially with respect to cardio toxicity (53). In comparison, the pulmonary (54) and tumor cytotoxicity (55) of bleomycin and the pulmonary toxicity of paraquat (56), agents believed to kill cells by generating oxyradicals, are enhanced when animals or tumor cells in culture are concomitantly exposed to elevated (>21%) oxygen levels. Thus, there is an experimental rationale for the expectation that hyperoxia would potentiate the cytotoxicity of Adriamycin. This expected result was observed with both the WT and the ADRR tumor cells, and the potentiation of Adriamycin cytotoxicity was more apparent with the sensitive line. The IC_{50} value for Adriamycin with the WT cells under hyperoxic conditions (about 3 nM) was approximately one third of the value under euoxic conditions, and considerably below the pharmacological plasma levels of Adriamycin in humans. Although there was a measurable increase in the toxicity of Adriamycin to the resistant cells at 10 \mu M drug, hyperoxia was not especially valuable in reversing the resistance to Adriamycin by these cells. Again, the most obvious explanation for these differences is attributed to the increased ability of the ADRR cells to detoxify superoxide and hydrogen peroxide by the actions of superoxide dismutase and selenium-dependent glutathione peroxidase.

There were clearly differences in the susceptibility of the sensitive and drug-resistant tumor cells to superoxide, hydrogen peroxide, hyperoxia, and especially Adriamycin, but both tumor lines were equally sensitive to gamma irradiation. Similar results were reported recently by Mitchell et al. (57), in that 30-fold daunomycin-resistant Chinese hamster ovary cells were inherently as radiosensitive as the parental line, even though the subline had approximately twofold higher levels of reduced glutathione, glutathione peroxidase, and catalase. An explanation for this apparent discrepancy may be that the damaging effects of ionizing irradiation were directed preferentially toward DNA. Any increased ability of the ADRR cells or Chinese hamster ovary cells to resist oxyradicals may have been bypassed because of the cytosolic localization of glutathione and glutathione peroxidase. Although superoxide, hydrogen peroxide, and hydroxyl radical are all produced by irradiation, the radiolysis of water can produce hydroxyl radical without superoxide and hydrogen peroxide intermediates (37). Neither superoxide dismutase nor glutathione peroxidase are capable of enzymatically scavenging hydroxyl radical. Alternatively, the "oxygen-fixation" hypothesis (58) suggests that hydroxyl radicals formed by ionizing radiation abstract protons from critical targets such as DNA bases to form secondary radicals. These target radicals then react with molecular oxygen to form peroxy radicals and other secondary oxidative products (58, 59). In this scenario, enzymatic defenses against superoxide or hydrogen peroxide would have no effect. This may explain why the ADRR tumor cells were as sensitive as the WT cells to irradiation, even though the ADRR cells were better protected against superoxide and hydrogen peroxide.

Results presented in this paper show the ADRR tumor cells to be resistant to oxyradicals, however, the degree of resistance toward superoxide or hydrogen peroxide differed markedly from the degree of resistance toward Adriamycin. We offer four possible interpretations of these observations. The first is that Adriamycin cytotoxicity is only partially mediated by the generation of oxyradicals, and other mechanisms of toxicity not only occur, but they may be additive and relatively more important than oxyradicals. The second is that Adriamycin-generated oxyradicals may be important in the cytotoxicity of the drug to WT tumor cells, especially because 10 to 20 nM concentrations of Adriamycin effectively kill most of the cells, and because WT cells generate much more hydroxyl radical than ADRR cells, at least at equivalent concentrations of Adriamycin in the range of 50 to 100 \mu M (16). By producing considerably more hydroxyl radical as the result of the redox cycling of Adriamycin, the WT tumors are killed by less than 10 nm drug; the ADRR tumor cells are not killed at these drug levels. Much higher concentrations of Adriamycin are necessary for equivalent cytotoxicity to the ADRR cells, on the order of 5 to 10 \mu M drug in continuous exposure, and it is possible that secondary or tertiary mechanisms become important as the extracellular concentration of Adriamycin is increased to these levels. Third, it is possible that Adriamycin redox cycles and generates oxyradicals at very specific sites in the tumor cells, perhaps within the mitochondria or even within the nucleus (60). Adriamycin juxtaposed to DNA would be expected to cause more critical damage than at other, less vital, locations. A fourth explanation is that oxyradical generation, or other pharmacological events in the WT tumors exposed to Adriamycin, initiated biochemical and genetic alterations that include increases in superoxide dismutase and glutathione peroxidase activities only as part of a catalogue of changes that collectively lead to markedly increased resistance to the drug. The cooperative functioning of all of the changes in the Adriamycin-selected resistant tumors, that is, the P-glycoprotein-mediated decreased net accumulation of Adriamycin, the increased ability to detoxify oxyradicals and secondary oxyradical products, and possibly other biochemical factors yet to be described such as differences in repair mechanisms or altered levels of targets, allow the ADRR tumor cells to survive in and even to slowly proliferate in 10 \mu M Adriamycin. The key to understanding the phenomenon of drug resistance may lie in elucidating those other factors.

In conclusion, breast tumor cells selected for acquired resistance to Adriamycin in culture have concurrently developed a tolerance to superoxide and hydrogen peroxide, most likely because of elevated activities of enzymatic defenses against oxyradicals. If the increased levels of enzymes that confer protection against oxyradicals in the drug-resistant tumor cells can be specifically inhibited, then at least a partial reversal of Adriamycin resistance might result, and the therapeutic benefit of Adriamycin might be improved.

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Differential Oxygen Radical Susceptibility of Adriamycin-sensitive and -resistant MCF-7 Human Breast Tumor Cells


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