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MOLECULAR PHARMACOLOGY, 46:1234-1240, 1994.

Doxorubicin, Daunorubicin, and Mitoxantrone Cytotoxicity in Yeast

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Received April 21, 1994; Accepted September 28, 1994

SUMMARY

We have investigated the effect of doxorubicin (Adriamycin) on the yeast *Saccharomyces cerevisiae*. Drug treatment was found to be cytotoxic to wild-type strains, in a concentration-dependent manner, whereas a petite mutant lacking the cytochrome oxidase (EC 1.9.3.1) subunit IV gene was resistant to doxorubicin. Transformation of the doxorubicin-resistant mutant with a yeast *in vivo* expression vector harboring the cytochrome oxidase subunit IV gene restored both respiration and sensitivity to doxorubicin. Another petite strain, with a mutation in the mitochondrial adenine nucleotide translocator (*pet9*), did not display doxorubicin resistance. However, in contrast to the subunit IV mutant, it possesses a functional respiratory chain. We also compared the cytotoxic effect of doxorubicin with those of daunorubicin and mitoxantrone in yeast. We found comparable levels of cytotoxicity for doxorubicin and daunorubicin, which were significantly greater than that for mitoxantrone. Finally, we constructed a yeast strain that overexpresses manganese superoxide dismutase (EC 1.15.1.1), an antioxidant enzyme present in mitochondria. Overexpression of manganese superoxide dismutase protected significantly against doxorubicin and daunorubicin cytotoxicity but only slightly against mitoxantrone cytotoxicity. Collectively, our results provide direct *in vivo* evidence that superoxide radicals participate in doxorubicin- and daunorubicin-induced cytotoxicity in yeast. Furthermore, these results indicate that mitochondrial respiration is a crucial factor in anthracycline, and perhaps mitoxantrone, cytotoxicity in yeast.

Doxorubicin (Adriamycin) is an anthracycline anticancer drug with an extremely broad spectrum of antitumor activity. Doxorubicin and the closely related anthracycline daunorubicin are very active against carcinomas of the lung, breast, stomach, and thyroid, as well as against both Hodgkin's and non-Hodgkin's lymphoma, several acute leukemias, and sarcomas (1, 2). Although several mechanisms have been proposed for this antitumor activity, an increasing body of evidence suggests an interaction of anthracycline agents with target cell DNA, causing inhibition of topoisomerase II activity (1, 3). Their broad spectrum of antitumor activity makes these drugs very useful chemotherapeutic agents. Although it has a narrower spectrum of activity, mitoxantrone, an anthracenedione derivative, maintains a pivotal role in cancer chemotherapy (4), in part due to its lesser cardiotoxicity (1). It is this cardiotoxicity that compromises the usefulness of doxorubicin and daunorubicin, limiting cumulative doses of these agents to 500 mg/m^2.

Chronic anthracycline-induced cardiotoxicity is characterized by myofibrillar degeneration, with damage to the sarcotubular system and mitochondria (5). Although several mechanisms have been suggested to explain this cardiotoxicity, a mechanism strongly supported by results from an array of experimental systems implicates the one-electron reduction of the anthracycline quinone to a semiquinone free radical, which subsequently reoxidizes in the presence of O_2, with generation of highly reactive oxygen radical species (1, 6). Evidence from several mammalian systems has demonstrated the production of semiquinone free radicals (7, 8), with resultant semiquinone-dependent $O_2 \cdot^-$, $\cdot OH$, and H_2O_2 generation (9, 10) and destructive lipid peroxidation (11).

Although free radical production may contribute to doxorubicin cardiotoxicity, several fundamental questions remain. For example, does free radical generation also account for the antitumor properties of the drug, which have been suggested to be mediated by topoisomerase II-dependent nuclear DNA damage (10)? Also, it has long been established that doxorubicin can intercalate into DNA and is found to be concentrated in nuclei of cells treated with the drug (1, 2). Are free radicals somehow involved in such a cytotoxic mechanism as well, or do different mechanisms operate simultaneously? It is quite possible that the cytosolic milieu of a given cell, be it in a tumor or cardiac cell, could dictate the particular mechanism of drug-induced cytotoxicity, such that different mechanisms exist in distinct cell types.

Although relatively few studies have employed the yeast *Saccharomyces cerevisiae* to investigate these cytotoxic effects, it has been demonstrated that resistance to doxorubicin is associated with a petite phenotype, which is characterized by a

This study was supported by Grant CH-495 from the American Cancer Society.

ABBREVIATIONS: COXIV, cytochrome oxidase subunit IV; SOD, superoxide dismutase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

dependence on glycolysis alone for cellular energy (12). Also, when wild-type yeast were treated with doxorubicin and the survivors were tested for the petite phenotype, the percentage of survivors that were petite increased as the drug concentration increased. Wild-type yeast strains typically contain a small percentage of cells that have spontaneously become petite. It was shown that these spontaneously petite cells in the wild-type population survived doxorubicin treatment; consequently, the drug was shown not to induce but, rather, to select for them.

In this report we present evidence demonstrating that a petite phenotype alone is not sufficient to impart doxorubicin resistance; mitochondrial respiration *per se* is required for the expression of doxorubicin-induced cytotoxicity in yeast. We also demonstrate the involvement of superoxide generation in drug-induced cytotoxicity and the differential participation of oxygen free radical species in anthracycline and anthracenedione cytotoxicity in yeast.

Materials and Methods

Yeast strains and plasmids. We used six *S. cerevisiae* strains. Strain COXIV– (MAT*a*, *leu2*, *his4*, *ade2*, *ura3*, *COXIV::LEU2*) was derived from strain JKR101 (MAT*a*, *leu2*, *his4*, *ade2*, *ura3*) by *COXIV* gene disruption (13). Strain D360–7D (MAT*a*, *pet9*, *his7*, *ade1*, *ade2*) (14) has a nonfunctional adenine nucleotide translocase. We constructed strain SOD+ by transforming JKR101 with the plasmid pFL141, which contains the complete yeast manganese SOD nucleotide sequence (15). Transformation of JKR101 with the pFL1 plasmid vector not containing the SOD insert yielded the control strain SOD–. Plasmid pmc4, a yeast vector carrying the *URA3* marker and a yeast centromere sequence, contains the yeast *COXIV* gene under the control of the yeast alcohol dehydrogenase I promoter (16). Transformation of strain COXIV– with plasmid pmc4 yielded strain COXIV+. Plasmids were propagated in the bacterial strain HB101 (17), and yeast transformation was performed as described (18).

Media for bacterial and yeast strain growth. Bacterial strains were grown in Luria broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride) supplemented with 50 μg/ml ampicillin. Yeast were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose), YPG medium (1% yeast extract, 2% peptone, 2% glycerol), or SDC medium (0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 0.65% ammonium sulfate, 40 μg/ml adenine, 40 μg/ml uracil). Plates for growing yeast included YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and YPEG plates (1% yeast extract, 2% peptone, 2% ethanol, 2% glycerol, 2% agar).

Drug treatments. Doxorubicin (Adriamycin RDF; manufactured by Farmitalia Carlo Erba S.p.A., Milan, Italy, and distributed by Adria Laboratories) stocks were prepared in a 0.9% sodium chloride solution containing 50 mg of lactose and 1 mg of methylparaben (added to enhance doxorubicin dissolution) per 10 mg of doxorubicin. Daunorubicin (Sigma Chemical Co.) stocks were prepared in distilled water. Mitoxantrone (Lederle Laboratories) was prepared in a solution containing 0.8% sodium chloride, 0.005% sodium acetate, and 0.046% acetic acid, pH 4. The stock solutions were filter sterilized and stored in aliquots at −70°. Yeast strains were all synchronized at G_0 by growth to saturation in YPD medium. Cells were then harvested by centrifugation and resuspended in distilled water. Cell suspensions were briefly sonicated to disperse cell aggregates and then diluted to 5×10^6 cells/ml in distilled water containing the indicated drug concentrations. Cells were then incubated for 100 min, with shaking at 300 rpm, at 30° in the dark. Cells were collected by centrifugation and resuspended in distilled water at appropriate dilutions before plating so that approximately 100 colonies/YPD plate resulted. Cell dilutions were plated in triplicate, plates were incubated for 48 hr at 30°, and then colonies were counted. The numbers of colonies formed at each drug concentration were compared with controls that were incubated in the absence of drugs (doxorubicin controls were incubated in the presence of the lactose/methylparaben solution in which doxorubicin stocks were prepared, whereas mitoxantrone controls were incubated in the presence of the same solution used for mitoxantrone stock preparation). All data points represent the mean of experiments repeated at least three times. Error bars represent standard errors. Experiments assessing the effect of cyanide upon doxorubicin toxicity in strain JKR101 used the same drug treatment protocol as outlined above, with the addition of a cyanide preincubation step before incubation with doxorubicin. After harvesting and sonication, cells were diluted in a 60 mM Tris solution, pH 9, containing either 50 mM sodium cyanide or distilled water (as a control). A 60-min incubation at 30°, with shaking at 300 rpm, in the dark followed before coincubation with doxorubicin.

SOD assay and measurement of respiratory rates. Yeast strains SOD+ and SOD– were grown overnight, to saturation, at 30° in YPD medium. Mitochondria were isolated as described (19) and were suspended in 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4. Samples were treated with 1% Triton X-100 to solubilize mitochondrial membranes for use in the activity assay. Protein concentrations of samples were determined using the Bio-Rad (Richmond, CA) dye reagent in the Bradford protein assay (20). SOD activity was measured in prepared mitochondria using a protocol based on the ferricytochrome *c* reduction assay (21, 22). This assay system measures the rate of reduction of cytochrome *c* by superoxide radicals generated by xanthine/xanthine oxidase and scavenged by SOD. One unit of SOD activity measured in the mitochondrial samples was defined as the amount of SOD needed to inhibit the rate of cytochrome *c* reduction by 50%, compared with the rate achieved in the presence of superoxide-generating xanthine/xanthine oxidase alone. Respiratory rates were determined in intact cells by polarigraphically measuring oxygen consumption.

Results

Doxorubicin cytotoxicity in wild-type and COXIV-deficient yeast strains. We initiated our investigation by comparing the effect of doxorubicin on the wild-type *S. cerevisiae* strain JKR101 and strain COXIV–, which was derived from JKR101 by disruption of the nuclear gene encoding COXIV. This subunit is required for cytochrome oxidase activity, and therefore COXIV– cells display no cyanide-sensitive oxygen consumption either *in vivo* or when their isolated mitochondria are analyzed (13). Both JKR101 and COXIV– were treated with increasing concentrations of doxorubicin in sterile water for 100 min, with shaking, at 30°. Sterile water maintains cells in G_0 and also minimizes nonspecific interactions between doxorubicin and growth medium components during the short term drug treatments. After drug treatment, the cells were reisolated in distilled water to remove unbound drug, diluted appropriately, plated onto YPD agar plates, and incubated for 48 hr at 30°. The resultant colonies, each derived from a single surviving cell after drug treatment, were then counted. The numbers of cells surviving each concentration of doxorubicin were subsequently compared with the numbers of cells present in a no-drug control, and the results are expressed as percentage survival (Fig. 1). As shown, COXIV– was much more resistant to doxorubicin than was JKR101. The difference in survival between these two strains is even greater than indicated in Fig. 1, because a significant percentage of the surviving JKR101 cells represent spontaneous petite mutants present in the starting population. This results in an overestimation of JKR101 survival, particularly at higher drug concentrations. By replica plating the JKR101 survivors onto YPEG plates, which require respiration for growth, we determined that the percentage of JKR101 survivors that are petite approaches 50% at 140 μg/ml doxorubicin (data not shown).

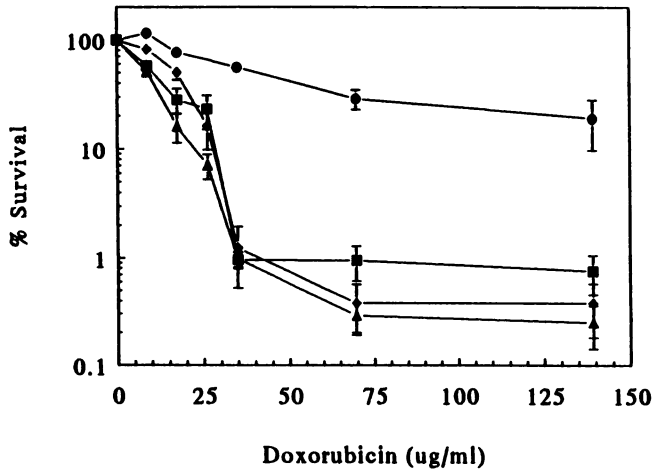

Fig. 1. Effect of doxorubicin treatment on yeast strains. Yeast strains were grown overnight (to saturation) at 30° in YPD medium, harvested by centrifugation, and resuspended at 5 × 10^6 cells/ml in distilled water containing the indicated concentrations of doxorubicin. Cells were then incubated for 100 min, with shaking at 300 rpm, at 30° in the dark, collected by centrifugation, and resuspended in distilled water. Aliquots were then plated in triplicate on YPD plates. Plates were incubated for 48 hr at 30° and then the colonies were counted. Results are expressed as percentage survival of cells exposed to the indicated drug concentration, compared with no-drug controls. All data points represent the mean of experiments repeated at least three times. *Error bars*, standard errors. ●, COXIV−; ▲, JKR101; ■, COXIV+; ♦, D360–7D.

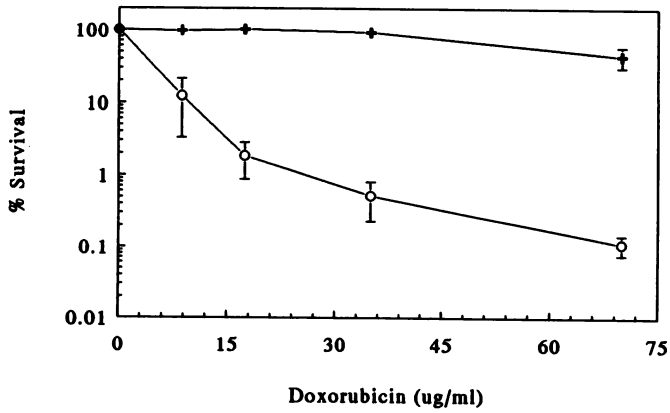

Fig. 2. Effect of cyanide on doxorubicin cytotoxicity. Yeast strain JKR101 was grown overnight (to saturation) at 30° in YPD medium, harvested by centrifugation, and resuspended at 5 × 10^6 cells/ml in 60 mM Tris, pH 9, containing 50 mM cyanide or distilled water (as a control). Cells were then incubated at 30° for 60 min, with shaking at 300 rpm, in the dark. The indicated concentrations of doxorubicin were then added, and the cells were incubated for 100 min, with shaking, at 30° in the dark, collected by centrifugation, and resuspended in distilled water. Aliquots were then plated in triplicate on YPD plates. Plates were incubated for 48 hr at 30° and then colonies were counted. Results are expressed as percentage survival of cells exposed to the indicated drug concentrations, compared with no-drug controls. All data points represent the means of experiments repeated at least three times, ± standard error. With cyanide; O, without cyanide.

To confirm the involvement of respiration in doxorubicin toxicity, we preincubated JKR101 in a sodium cyanide solution and determined the subsequent survival after coincubation with doxorubicin. As seen in Fig. 2, cyanide, an inhibitor of cytochrome oxidase, conferred significant resistance to JKR101 when cells were exposed to it before doxorubicin.

We considered that the difference in sensitivity to doxorubicin cytotoxicity between COXIV− and JKR101 might result from a difference in their growth rates or in their ability to recover and grow after incubation in distilled water during drug treatment. However, the growth rates of these strains were virtually identical (Fig. 3), as was their ability to recover after incubation for 100 min in distilled water at 30° (Fig. 4). Importantly, in no-drug controls for both COXIV− and JKR101, cell number did not increase during incubation in distilled water for up to 2 hr, nor did cell viability decrease after incubation for 2 hr in distilled water (data not shown).

As detailed previously, COXIV− was derived from JKR101 by disruption of the *COXIV* gene. Respiratory-deficient mutants such as COXIV− may, however, become defective in other respiratory proteins, because they cannot be propagated on media requiring respiration for survival. To ensure that the only difference, in terms of respiration, between COXIV− and JKR101 is the absence of functional COXIV, we introduced this gene into COXIV− by transforming these cells with the plasmid pmc4, which harbors the intact *COXIV* gene. The resulting transformant, COXIV+, grew well on glycerol, indicating that the absence of COXIV in COXIV− constituted the only respiratory chain mutation. When COXIV+ was tested for its response to doxorubicin treatment (Fig. 1), it was clear,

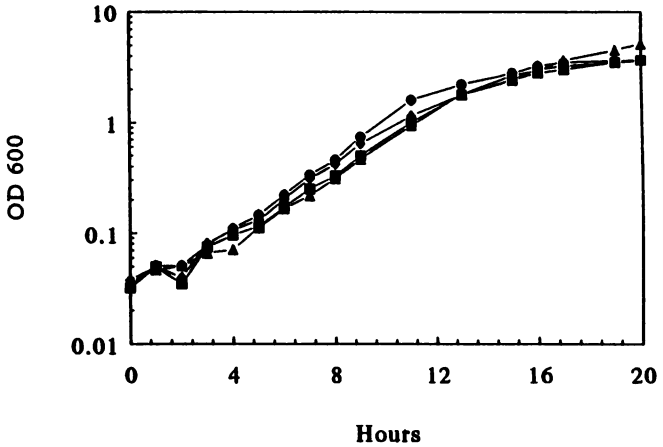

Fig. 3. Growth of yeast in YPD medium. Yeast strains were inoculated from growing precultures into liquid YPD medium. Cell growth was measured by absorbance at 600 nm. ●, COXIV−; ▲, JKR101; ■, COXIV+; ♦, D360–7D.

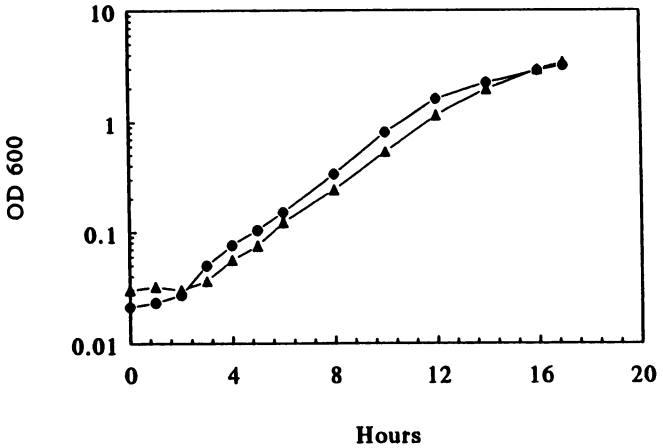

Fig. 4. Growth recovery after 100-min incubation under nongrowing conditions. Strains COXIV− (●) and JKR101 (▲) were incubated for 100 min, with shaking, at 30° in distilled water. The cells were then recovered by centrifugation, resuspended in distilled water, and inoculated into liquid YPD medium. Cell growth was followed by measuring absorbance at 600 nm.

upon comparison with COXIV−, that it had regained drug sensitivity. Consequently, these data clearly indicate that COXIV is required for doxorubicin cytotoxicity in yeast.

Evidence that a petite phenotype alone is insufficient to impart doxorubicin resistance in yeast. The resistance of petite cells to doxorubicin is consistent with the notion that mitochondrial respiration is crucial for drug cytotoxicity in yeast. However, perhaps respiration *per se* is not critical but, rather, drug resistance depends upon an undefined aspect of the petite phenotype. To address this issue, we attempted to identify a yeast strain that was petite but sensitive to doxorubicin. Strain D360–7D, which contains a mutation (*pet9*) in the adenine nucleotide translocator (14, 23), displayed these characteristics. D360–7D is unable to transport ADP in exchange for ATP and hence cannot rely upon oxidative phosphorylation for energy. However, as shown in Fig. 1, strain D360–7D exhibited doxorubicin sensitivity similar to that of the wild-type strain JKR101. We tested D360–7D cells for respiration and observed that they displayed significant cyanide-sensitive oxygen consumption (data not shown). Therefore, although D360–7D is petite with respect to its carbon source requirements, its respiratory chain is functional. It is consequently clear that the petite phenotype alone cannot impart doxorubicin resistance in yeast; mitochondrial respiration *per se* is a crucial factor.

Cytotoxicity of doxorubicin, daunorubicin, and mitoxantrone in yeast strains JKR101 and COXIV−. We compared the survival of the wild-type strain JKR101 in response to treatment with increasing concentrations of doxorubicin, daunorubicin, and mitoxantrone. As shown in Fig. 5, doxorubicin and daunorubicin induced similar levels of cytotoxicity in JKR101, whereas mitoxantrone was significantly less cytotoxic. These results are in agreement with others previously reported, wherein mitoxantrone was observed to cause diminished levels of cytotoxicity, in comparison with doxorubicin, in a chronic cardiotoxicity mouse model (24).

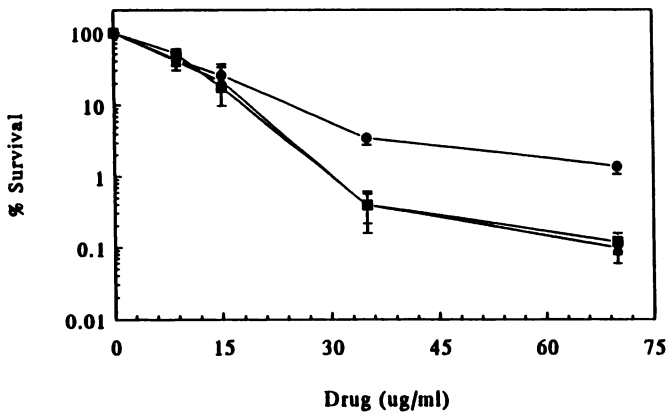

Fig. 5. Comparison of doxorubicin, daunorubicin, and mitoxantrone cytotoxicity in JKR101. Yeast were grown overnight (to saturation) at 30° in YPD medium, harvested by centrifugation, and resuspended at 5 × 10^6 cells/ml in distilled water containing the indicated drug concentrations. Cells were then incubated for 100 min, with shaking at 300 rpm, at 30° in the dark, collected by centrifugation, and resuspended in distilled water. Aliquots were then plated in triplicate on YPD plates. Plates were incubated for 48 hr at 30° and then the colonies were counted. Results are expressed as percentage survival of cells exposed to the indicated drug concentrations, compared with no-drug controls. All data points represent the mean of experiments repeated at least three times. *Error bars*, standard errors. ▲, Doxorubicin; ■, daunorubicin; ●, mitoxantrone.

Fig. 6 shows that doxorubicin (Fig. 6, *upper*), daunorubicin (Fig. 6, *middle*), and mitoxantrone (Fig. 6, *lower*) were each less cytotoxic to the COXIV-deficient yeast strain COXIV−, compared with the wild-type JKR101. From these data it is clear that a functional respiratory chain is involved in doxorubicin-,

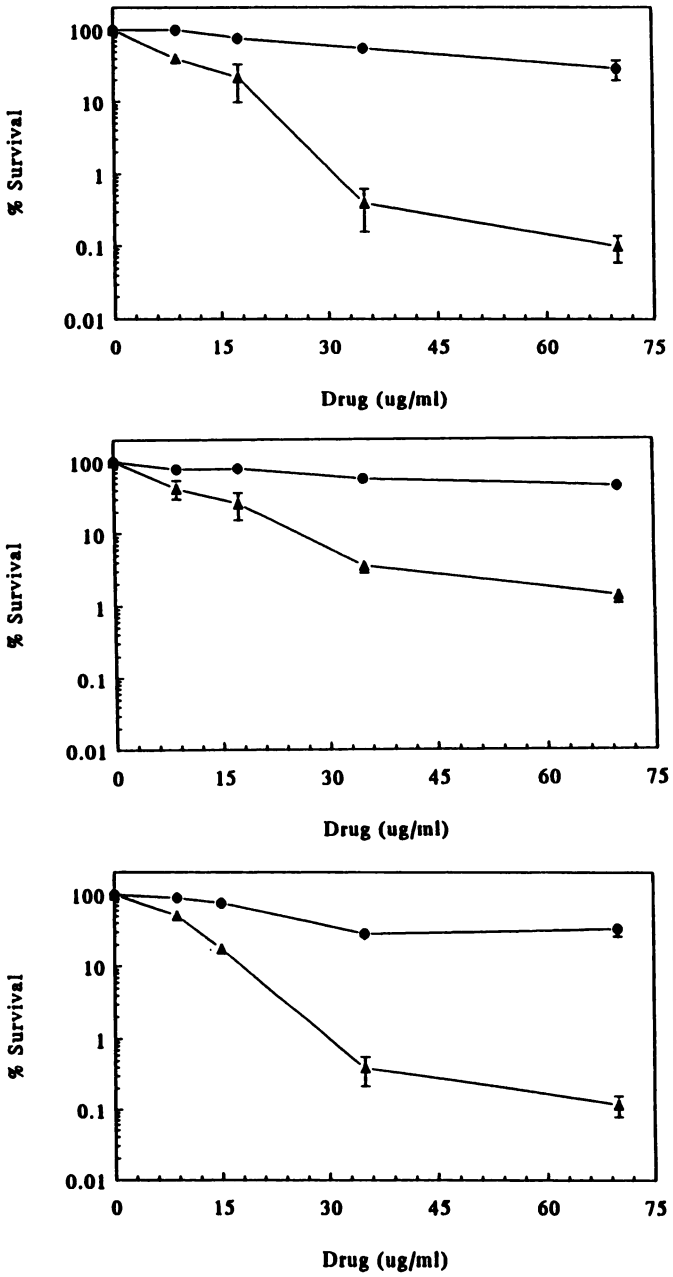

Fig. 6. Comparison of doxorubicin, daunorubicin, and mitoxantrone toxicity in JKR101 and COXIV−. Yeast strains were grown overnight (to saturation) at 30° in YPD medium, harvested by centrifugation, and resuspended to 5 × 10^6 cells/ml in distilled water containing the indicated concentrations of doxorubicin, daunorubicin, or mitoxantrone. Cells were then incubated for 100 min, with shaking at 300 rpm, at 30° in the dark, collected by centrifugation, and resuspended in distilled water. Aliquots were then plated in triplicate on YPD plates. Plates were incubated for 48 hr at 30° and then colonies were counted. Results are expressed as percentage survival of cells exposed to the indicated drug concentrations, compared with no-drug controls. All data points represent the means of experiments repeated at least three times, ± standard error. *Upper*, doxorubicin; *middle*, daunorubicin; *lower*, mitoxantrone. ▲, JKR101; ●, COXIV−.

daunorubicin-, and mitoxantrone-induced cytotoxicity in yeast. This result suggests a dependence of drug cytotoxicity on mitochondrial respiration and perhaps radical oxygen species generation.

Comparison of resistance to doxorubicin, daunorubicin, and mitoxantrone cytotoxicity between strains overexpressing manganese SOD. Fig. 7 demonstrates that strain SOD+, which overexpresses manganese SOD, was significantly more resistant to both doxorubicin-induced (Fig. 7, *upper*) and daunorubicin-induced (Fig. 7, *middle*) cytotoxicity than was the control strain SOD−. Manganese SOD overexpression afforded little protection, however, against mitoxantrone-induced cytotoxicity at any concentration tested (Fig. 7, *lower*). These results suggest that $O_2\cdot^-$ production plays a critical role in doxorubicin- and daunorubicin-induced cytotoxicity in yeast but may be of only marginal significance in mitoxantrone cytotoxicity.

To confirm that the enhanced drug resistance of SOD+ is not attributable to inherent variations in growth characteristics, in comparison with SOD−, we determined the growth and respiratory rates of these two strains. The growth rates of SOD+ and SOD− are depicted in Fig. 8. The two strains grew at similar rates with both a fermentable (YPD medium) (Fig. 8, *upper*) and a nonfermentable (YPG medium) (Fig. 8, *lower*) carbon source, indicating that overexpression of manganese SOD does not affect the rate of cell growth or the ability to respire and thereby grow on a nonfermentable carbon source that requires functional mitochondrial respiration for growth. Furthermore, the rates of oxygen consumption measured polarigraphically for the two strains were also very similar (data not shown). Although the growth and respiratory rates of SOD+ and SOD− were quite similar, their manganese SOD activities differed noticeably, with values of 96.53 units/mg of mitochondrial protein and 52.23 units/mg of mitochondrial protein, respectively. The endogenous SOD activity reported here compares favorably with that of 42 units/mg of mitochondrial protein reported previously for wild-type *S. cerevisiae* (25).

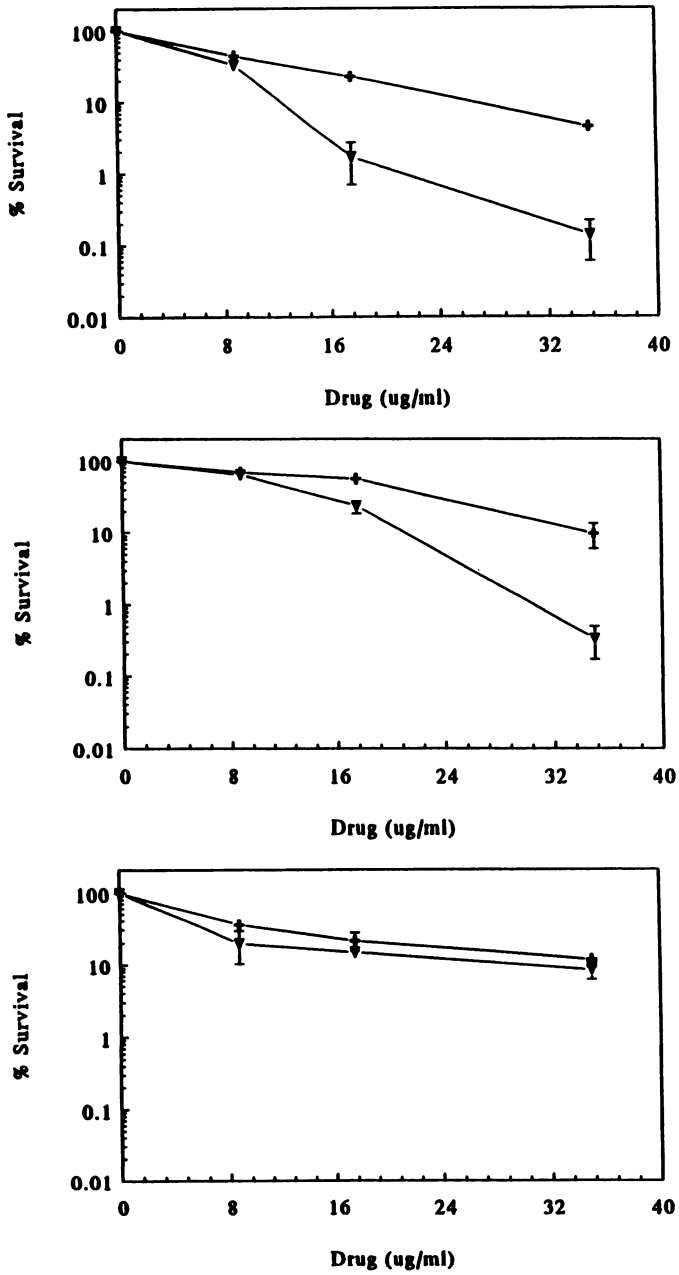

Fig. 7. Comparison of doxorubicin, daunorubicin, and mitoxantrone cytotoxicity in SOD+ and SOD−. Experimental conditions were the same as those described in the legend to Fig. 5. Results are expressed as percentage survival of cells exposed to the indicated drug concentrations, compared with no-drug controls. *Upper*, doxorubicin; *middle*, daunorubicin; *lower*, mitoxantrone. +, SOD+; ▼, SOD−.

Discussion

The anticancer agents doxorubicin, daunorubicin, and mitoxantrone are established components of tumor therapy.

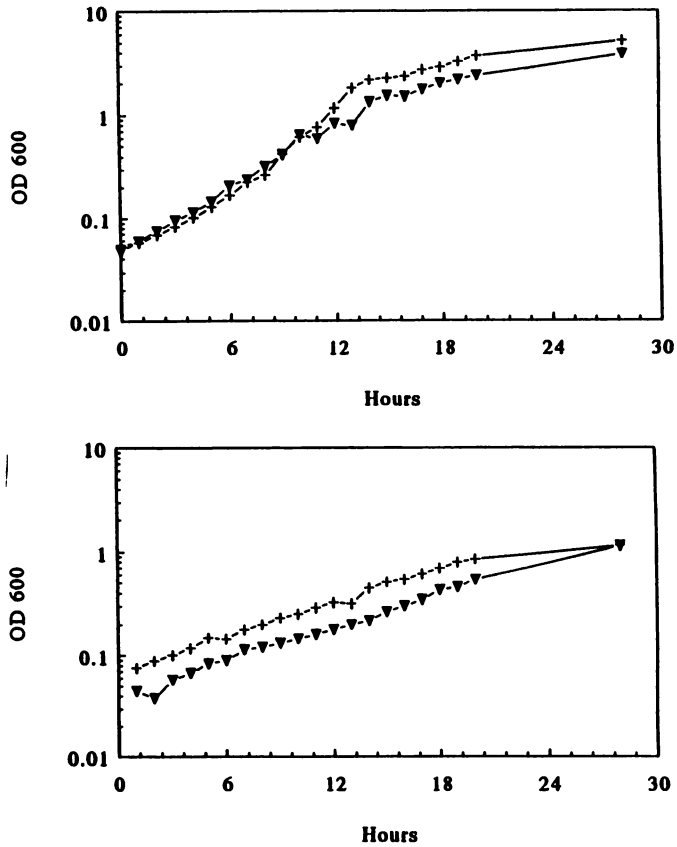

Fig. 8. Growth of yeast strains SOD+ and SOD− in YPD and YPG media. Yeast strains SOD+ and SOD− were inoculated from growing precultures into liquid YPD medium (*upper*) or liquid YPG medium (*lower*). Cell growth was measured by absorbance at 600 nm. +, SOD+; ▼, SOD−.

However, their applicability is severely limited by the dose-dependent cardiotoxicity they induce (primarily doxorubicin and daunorubicin). In the experiments reported here, we sought to better understand the mechanism of this cytotoxicity by utilizing several strains of the yeast *S. cerevisiae* that exhibit a range of phenotypes with respect to mitochondrial respiration, carbon source requirements, and antioxidant enzyme expression.

As mentioned previously, there is a growing body of evidence for an oxygen radical-mediated mechanism of anthracycline cardiotoxicity. One-electron reduction of doxorubicin and daunorubicin to their semiquinone structures by the NADH dehydrogenase complex [NADH:(acceptor) oxidoreductase, EC 1.6.99.3] has been detected using ESR spectroscopy of heart submitochondrial particles (8). The sarcosomal NADPH-cytochrome P450 reductase (EC 1.6.2.4) and cytosolic xanthine oxidase (EC 1.2.3.2) also appear to reduce doxorubicin in their respective rat heart cell fractions (9). Subsequent electron transfer from anthracycline semiquinones to oxygen, with resultant formation of $O_2\cdot^-$, H_2O_2, and $\cdot OH$, has also been demonstrated in cardiac submitochondrial particles (10) and perfused rat heart (26). Anthracycline-induced lipid peroxidation in cardiac tissues and organelle preparations (11, 27), resulting from the interaction of reactive oxygen species, possibly $\cdot OH$, with membrane lipids, further supports a role for free radical generation in anthracycline cardiotoxicity.

Although such evidence certainly suggests a mechanism of cardiotoxicity involving reactive oxygen species and subsequent oxidative damage of cardiac cellular constituents, this mechanism is by no means universally accepted or definitively confirmed. Difficulty in detecting radical oxygen species (26), especially *in vivo* (1), and the inability of several free radical scavengers to consistently attenuate anthracycline-induced cardiotoxicity (5) have contributed to criticism of the free radical hypothesis. To address these concerns, we used the yeast *S. cerevisiae* as an experimental system in which we could readily manipulate the expression of various proteins to alter the ability of the cells to support or prevent the formation of radical oxygen species in response to anticancer agent insult.

In our experiments, we demonstrated that mitochondrial respiration is required for anthracycline-induced cytotoxicity in yeast. Disruption of the *COXIV* gene conferred significant resistance to doxorubicin cytotoxicity. Acquisition of drug sensitivity by transformation of COXIV− with the *COXIV* gene confirmed that this critical component of the electron transport chain is required for doxorubicin-induced cytotoxicity in yeast. Further confirmation of the role of respiration and cytochrome oxidase activity in doxorubicin cytotoxicity was provided by pretreatment of strain JKR101 with the cytochrome oxidase inhibitor cyanide before doxorubicin treatment. Inhibition of cytochrome oxidase, and hence respiration, by cyanide yielded enhanced survival similiar to that conferred by *COXIV* gene disruption observed in strain COXIV−. The use of this electron transport chain inhibitor thus provides additional support to supplement the evidence derived from our genetic studies using the mutant yeast strain COXIV−, which demonstrated enhanced resistance to doxorubicin cytotoxicity by disruption of the *COXIV* gene. Perhaps loss of cytochrome oxidase activity causes an inhibition of electron flow, which results in reduction of upstream respiratory chain elements to redox states or structures incapable of donating electrons to the quinone moiety of doxorubicin. Similar reasoning was used to explain the inability of cytochrome *c* oxidase-deficient *S. cerevisiae* mitochondria to generate $O_2\cdot^-$ by coenzyme Q-mediated O_2 reduction (28). In addition to implicating mitochondrial respiration as a prerequisite for doxorubicin cytotoxicity in yeast, our experiments also demonstrate that expression of the petite phenotype alone does not confer drug resistance. This extends previous results that reported an association of drug resistance with a petite phenotype (12) that was selected, not induced, by doxorubicin treatment.

Implications of mitochondrial respiration in anthracycline cytotoxicity in yeast provide preliminary evidence for oxygen radical involvement in this cytotoxicity. To further delineate the role of oxygen and its reduction to reactive species, we constructed a yeast strain, SOD+, that overexpressed manganese SOD, a mitochondrial protein that catalyzes the conversion of $O_2\cdot^-$ to H_2O_2. Overexpression of SOD conferred doxorubicin and daunorubicin resistance, indicating that $O_2\cdot^-$ participates in the cytotoxicity of these two drugs in yeast. $O_2\cdot^-$ would theoretically result from one-electron reduction of oxygen by the anthracycline semiquinone (1). The implications of our results agree with previous reports that demonstrated $O_2\cdot^-$ generation from anthracyclines in mammalian systems (9–11). $O_2\cdot^-$ can then react with itself, nonenzymatically or in a reaction catalyzed by SOD, to yield H_2O_2. H_2O_2 can be subsequently reduced by $O_2\cdot^-$, by semiquinones, or in an Fe^{2+}-catalyzed reaction to generate $\cdot OH$, a very destructive and reactive chemical species capable of damaging DNA and membranes (1). $O_2\cdot^-$, which directly inactivates certain enzymes, and H_2O_2, which damages the cell by sulfhydryl group oxidation or generation of high oxidation states, are also destructive radical species. The fact that manganese SOD overexpression confers enhanced resistance to anthracycline-induced cytotoxicity does not allow us to definitively identify which reactive oxygen species ($O_2\cdot^-$, H_2O_2, or $\cdot OH$) actually elicits cellular damage. We can, however, conclude that $O_2\cdot^-$ participates at some level in mediating doxorubicin- and daunorubicin-induced cytotoxicity in yeast. Future experiments using strains overexpressing other antioxidant enzymes, such as catalase, may assist us in pinpointing the mechanism of anthracycline-induced cytotoxicity in yeast.

Although $O_2\cdot^-$ appears to participate in doxorubicin and daunorubicin cytotoxicity in yeast, it is apparently not as critical in mitoxantrone cytotoxicity, because overexpression of manganese SOD has little effect on mitoxantrone resistance (Fig. 7, *lower*). Mitoxantrone is an anthracenedione derivative that has a narrower spectrum of antitumor activity, compared with the anthracyclines, but produces significantly less cardiotoxicity (1), which does occur to a lesser extent (24). Our observation that SOD overexpression does not increase resistance to mitoxantrone may result from mitoxantrone-dependent $O_2\cdot^-$ generation at a cellular site distant from mitochondria and hence unavailable for detoxification by the mitochondrially localized manganese SOD. On the other hand, mitoxantrone has been demonstrated to damage mitochondria coincidentally with its moderate toxicity to cardiac cells (24). The highly reactive nature of oxygen radical species, which necessitates their production at the site of their cellular damage (3), and the short diffusion radius of the semiquinone radical (6) appear to exclude the possibility that mitoxantrone generates $O_2\cdot^-$ at a cellular site distant from mitochondria. Published results indicate that mitoxantrone, in comparison with anthracyclines, generates fewer (if any) oxygen free radicals (29) and inhibits,

rather than stimulates, lipid peroxidation (30). Even though mitoxantrone elicits a free radical signal, this occurs by a mechanism different from that of anthracyclines (31). Collectively, these reports demonstrate that mitoxantrone, in contrast to the anthracyclines, does not appear to generate substantial reactive oxygen species, which may contribute to its lesser cardiotoxicity. There exists the possibility that the unique free radical signal of mitoxantrone may, via hydroperoxy fatty acids, affect mitochondrial and sarcotubular calcium levels, thereby damaging these organelles and causing limited drug-induced cardiotoxicity (24, 32). Our results (Fig. 6, *lower*) demonstrate that, indeed, mitoxantrone cytotoxicity in yeast does depend on a functional respiratory chain. Perhaps reduction of mitoxantrone by the mitochondrial respiratory chain forms a nitrogen-centered free radical that could elicit the calcium disturbance and organellar disruption described above.

In conclusion, we have shown that doxorubicin and daunorubicin, and to a lesser extent mitoxantrone, require mitochondrial respiration for cytotoxicity in the yeast *S. cerevisiae* and that a petite phenotype alone is not sufficient to impart resistance to this cytotoxicity. Furthermore, overexpression of manganese SOD confers resistance to doxorubicin and daunorubicin cytotoxicity but not that of mitoxantrone, thereby implicating $O_2\cdot^-$ participation in the cytotoxic mechanism of the two anthracyclines. We anticipate future use of this versatile eukaryotic *in vivo* system to further delineate the precise mechanism and cellular reactions mediating anthracycline-induced cytotoxicity, for comparison with similar events occurring in cardiac cells of higher eukaryotes.

Acknowledgments

We thank Drs. Anita Hopper, Carol Whitfield, and Jim Hopper for critically reviewing the manuscript. We are especially grateful to Dr. B. D. Lemire, University of Alberta, for many valuable discussions and for providing the yeast strain COXIV−. Adriamycin RDF was a gift from Adria Laboratories (Columbus, OH). We also thank M. B. Verner and M. Yablonski for excellent technical help.

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