

Prevention of Doxorubicin Cardiac Toxicity in the Mouse by *N*-Acetylcysteine

JAMES H. DOROSHOW, GERSHON Y. LOCKER, INA IFRIM, and CHARLES E. MYERS,
*Division of Medical Oncology, Department of Medicine and Cancer Center,
University of Southern California School of Medicine, Los Angeles, California
90033; Division of Medical Oncology, St. Joseph Hospital, Chicago, Illinois 60657;
Arthur D. Little, Inc., Cambridge, Massachusetts 02166; and Clinical
Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute,
Bethesda, Maryland 20205*

ABSTRACT This study was undertaken to investigate the effect of exogenous sulfhydryl compound administration on the toxicity of doxorubicin in mice. Pretreatment of CDF₁ mice with a pharmacologic dose (2,000 mg/kg) of *n*-acetyl-L-cysteine 1 h before doxorubicin (20 mg/kg, i.p.) decreased lethality from 100% ($n = 44$) to 37.7% ($n = 53$), $P < 0.001$. Variation in the timing and dose of *n*-acetylcysteine significantly diminished its protective activity. Pretreatment with *n*-acetylcysteine also significantly reduced long-term mortality in animals receiving multiple doses of doxorubicin; 10 wk after the third of three doxorubicin doses (5 mg/kg, i.p.) administered at 2-wk intervals, survival in the *n*-acetylcysteine pretreated group was 51.4% ($n = 35$) compared with 16.7% ($n = 30$) for animals receiving saline before doxorubicin, $P < 0.01$. In this experiment, *n*-acetylcysteine pretreatment also diminished doxorubicin-related losses in total body weight and heart wet weight by 55.2% ($P < 0.05$), and 60.9% ($P < 0.02$), respectively, compared with animals pretreated with saline.

N-acetylcysteine pretreatment also ablated electron microscopic evidence of doxorubicin cardiomyopathy without alleviating morphological features of its toxic effects on the liver or small intestinal mucosa. The cardioprotective action of *n*-acetylcysteine may be par-

tially explained by the $429 \pm 60\%$ increase in cardiac nonprotein sulfhydryl content ($P < 0.01$) that was measured one hour after *n*-acetylcysteine administration; nonprotein sulfhydryl concentration in the liver at the same time was insignificantly different from control levels. Treatment with *n*-acetylcysteine also increased the nonprotein sulfhydryl content of P388 leukemia cells nearly threefold; however, it did not alter the chemotherapeutic activity of doxorubicin against this murine tumor. Whereas *n*-acetylcysteine blocked doxorubicin cardiac toxicity, it did not affect the uptake or metabolism of doxorubicin in the heart or liver.

These results suggest that the concentration of free sulfhydryl groups in the heart may play a role in the development of doxorubicin cardiac toxicity and that augmenting cardiac nonprotein sulfhydryl group content with *n*-acetylcysteine may provide a means to enhance the chemotherapeutic index of doxorubicin.

INTRODUCTION

Doxorubicin is an antineoplastic antibiotic that is now part of standard chemotherapeutic regimens for most hematopoietic malignancies as well as for advanced solid tumors of the breast, ovary, thyroid, and bone (1). Unfortunately, treatment with this agent is limited by a potentially lethal and dose-dependent congestive cardiomyopathy, which clinical and pathological studies have recently shown may occur soon after drug therapy has begun (2, 3).

Although the mechanism of doxorubicin cardiac toxicity is incompletely understood, several reports suggest that it may result from unrestrained, drug-induced, cardiac reactive oxygen metabolism (4-7). It is now known that certain flavin-containing enzymes convert doxorubicin *in vitro* to a semiquinone free

This work was presented in preliminary form at the 1978 Annual Meeting of the American Federation for Clinical Research and at the 1979 Annual Meeting of the American Association for Cancer Research; abstracts pertaining to this work were published in 1978. (*Clin. Res.* 26: 434A) and in 1979. (*Proc. Am. Assoc. Cancer Res.* 20: 253).

Dr. Doroshow's current address is Pharmacology Section, Department of Medical Oncology, City of Hope National Medical Center, Duarte, Calif. 91010.

Received for publication 30 October 1980 and in revised form 17 June 1981.

radical; the cyclical oxidation and reduction of this drug radical catalyzes electron flow from reduced pyridine nucleotides to molecular oxygen (6). Recent investigations by Thayer (7) and by our laboratory (8) have shown that electron transfer after treatment with doxorubicin is markedly enhanced in the heart, and leads to a substantial increase in superoxide anion and hydrogen peroxide formation in mitochondria and sarcoplasmic reticulum, two major sites of cardiac damage from doxorubicin (9). Furthermore, the cytotoxic potential of this cardiac oxygen radical production may be magnified by the inhibitory effect of doxorubicin on the heart's limited enzymatic capacity to detoxify reactive oxygen metabolites (10).

The accumulation of drug-induced reactive oxygen radicals in heart cells may explain the cardiac lipid membrane peroxidation (4) and the enhanced hexose monophosphate shunt activity that accompanies treatment with doxorubicin (11). A drug-related oxygen radical cascade could also explain the depletion of cardiac reduced glutathione pools that have been found after doxorubicin administration (12, 13, 14). These studies, and our previous finding that alterations in the glutathione-glutathione peroxidase system significantly increase doxorubicin toxicity (10), suggested that the free sulfhydryl content of the heart may be a major determinant of the cardiac toxicity of doxorubicin. Since sulfhydryl groups play an important role in the maintenance of muscular contractile function (15) and membrane integrity (16), and also promote the nonenzymatic detoxification of hydroxyl radicals (17) and lipid peroxides (18, 19), we hypothesized that augmenting cardiac sulfhydryl group content might enhance the ability of heart muscle to withstand doxorubicin exposure. Because reactive oxygen production seems to play a lesser role in the chemotherapeutic activity of doxorubicin (4, 20), we proposed that increasing the sulfhydryl content of the heart might selectively improve the therapeutic index of the drug.

Our results indicate that treatment of experimental animals with pharmacologic concentrations of the sulfhydryl *n*-acetylcysteine before doxorubicin administration produces a fourfold increase in cardiac nonprotein sulfhydryl levels while eliminating electron microscopic features of doxorubicin cardiac toxicity. In identical dosages, *n*-acetylcysteine does not interfere with the antineoplastic activity of doxorubicin or with the pharmacokinetics of doxorubicin uptake in the heart or liver.

METHODS

Experimental animals. For these experiments, male CDF₁ mice were obtained at weaning from Dublin Laboratories, Dublin, Va. The mice were raised on National Institutes of Health rat and mouse ration No. 5108 as pre-

viously described (10); were housed in a constant (22°C) temperature environment with alternating 12-h wake-sleep cycles; and were caged on hardwood bedding and given tap water ad lib. Experimental studies were performed on these animals after 6 wk of maturation.

Materials. Doxorubicin hydrochloride for injection that was of clinical grade was obtained from the Investigational Drug Branch, Division of Cancer Treatment, National Cancer Institute. Doxorubicin hydrochloride was reconstituted in 0.85% sterile sodium chloride on the day of administration and was protected from light until used. *N*-acetyl-L-cysteine (NAC),¹ glutathione (reduced form, GSH), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), tris (hydroxy-methyl) amino-methane (Tris), EDTA, and diagnostic reagents for the determination of glutamic-pyruvic transaminase were obtained from Sigma Chemical Co., St. Louis, Mo. Silver nitrate, sodium chloride, sodium hydroxide, oxalic acid, trichloroacetic acid (all American Chemical Society reagent grade) as well as methanol (spectral grade), *n*-butanol (spectral grade), isopropanol, and chloroform were purchased from Fisher Scientific Co., Pittsburgh, Pa. Silica gel plates (Silica gel 60, precoated plate) were obtained from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.

Animal studies. In these studies, experimental animals were housed 10 to a cage. Doxorubicin was administered by intraperitoneal injection in a constant volume of saline. Where indicated, control animals were treated simultaneously with identical volumes of 0.85% sterile sodium chloride. Doxorubicin (or saline) treatments consistently occurred at the same time of day to avoid possible inconsistencies produced by diurnal variations in tissue nonprotein sulfhydryl levels (12). NAC was prepared on ice in 0.85% sterile sodium chloride, neutralized to pH 7.0 with 1 N sodium hydroxide, and routinely administered to unanesthetized mice either by gastric intubation or by intraperitoneal injection of a constant volume of drug in saline. Animals not receiving NAC were treated by similar routes of administration with equivalent dosages of saline alone.

During our preliminary examination of the protective effect of NAC on the toxicity of a single dose of doxorubicin, mice were pretreated with either NAC or saline 1 h before receiving doxorubicin; control animals were pretreated similarly but subsequently received saline instead of doxorubicin. This format was used to determine dose-response relationships for NAC and to assess the effectiveness of different routes of NAC administration. The optimal timing for NAC dosage with respect to doxorubicin treatment was investigated at the highest NAC dose used in these studies (2,000 mg/kg body wt). Survival studies in some instances represent the pooled data from multiple identical experiments. In all of these experiments, treatment groups were followed for 8 wk after injection to determine both short- and long-term effects of NAC on doxorubicin toxicity.

To assess the effect of NAC pretreatment on the chemotherapeutic activity of doxorubicin, mice were implanted intraperitoneally, as previously described (4), with 1 million P388 leukemia cells after cell viability (routinely >95%) had been confirmed by exclusion of 0.1% trypan blue dye; 24 h after implantation, NAC (2,000 mg/kg body wt) or an equal volume of sterile physiologic saline was administered by intraperitoneal injection. 1 h following pretreatment with NAC, the mice received either doxorubicin or an equi-

¹Abbreviations used in this paper: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GPT, glutamic-pyruvic transaminase; GSH, reduced glutathione; NAC, *n*-acetyl-L-cysteine; NPSH, nonprotein sulfhydryl.

volume dosage of physiologic saline intraperitoneally. Experiments were performed using two dosages of doxorubicin, 7.5 and 10 mg/kg body wt. Experimental animals pre-treated with saline were handled similarly to those receiving NAC. Each of the experimental groups was comprised of at least 30 animals; these mice were also followed for 8 wk after treatment unless all the animals in a group had died earlier from uncontrolled intraperitoneal malignancy.

For studies of the effect of NAC on the toxicity of multiple doses of doxorubicin, experimental animals were treated intraperitoneally with 5 mg/kg doxorubicin or an equal volume of physiologic saline every 2 wk for a total of three doses (cumulative doxorubicin dose 15 mg/kg body wt). 1 h before each doxorubicin or saline injection, NAC (2,000 mg/kg body wt) or an equivalent volume of physiologic saline was administered intraperitoneally. Body weight was measured before each injection and intermittently thereafter. The animals were killed 10 wk after the last drug treatment, or a total of 14 wk from the beginning of the study; heart wet weight was determined in all surviving doxorubicin-treated animals and in animals receiving saline or NAC only.

Nonprotein sulfhydryl levels. Nonprotein sulfhydryl group levels in mouse heart and liver were measured by modification of the method described by Sedlak and Lindsay (21). To determine tissue NPSH concentrations after treatment with NAC, three experimental animals per time point were injected intraperitoneally with an NAC dose of 2,000 mg/kg body wt between 8 and 9 a.m. PDST. An equal number of control animals received an equivalent dose of physiologic saline intraperitoneally at the same time. Subsequently, experimental and control animals were killed by cervical dislocation; ventricles for cardiac nonprotein sulfhydryl (NPSH) levels were excised, trimmed of excess connective tissue, blotted dry, and weighed immediately. The left hepatic lobe was also employed for NPSH determination and was prepared similarly. Throughout the assay procedure each sample, which consisted of the ventricular tissue or left hepatic lobe of one mouse, was regularly kept on melted ice. Tissues were vigorously washed free of erythrocytes at least four times with an iced solution of 20 mM EDTA prepared with deionized, glass distilled water, and then homogenized at 4°C for 30 s in 4 ml of the EDTA solution with a Brinkmann model PCU-2-110 Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.). We have previously shown that after our vigorous washing procedure only 5% or less of the total protein in the tissue sample can be accounted for by hemoglobin (10). In the cold, 2.0 ml of deionized, glass distilled water and 0.5 ml of 50% (wt/vol) trichloroacetic acid were added to a 2.5 ml sample of the homogenate. The mixture was covered and then shaken vigorously in the cold for 15 min. The samples were then centrifuged at 3,000 g and 4°C for 15 min. 1 ml of the resulting supernate was added to 2.0 ml of 0.4 M Tris (pH 8.9); after mixing, 0.1 ml of 0.01 M DTNB was added to each sample; the samples were vigorously mixed again and the relative NPSH concentrations were determined within 3 min in a 1-ml volume by spectrophotometric measurement at 412 nm and 25°C with a Gilford Model 250 recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). In these experiments, we used a 4-ml vol of 20 mM EDTA that had been completely processed with the other tissue samples as the blank in our spectrophotometric measurements. We also examined the optical density of our tissue samples at 412 nm before the addition of DTNB; in no instance did this exceed 0.008 absorbance units.

We also investigated the effect of treatment with NAC on the NPSH concentration of P388 leukemia cells *in vivo*. Mice were implanted with one million P388 leukemia cells as previously described. 6 d after implantation, 12 groups of three

animals each were treated with either NAC (2,000 mg/kg) or an equivalent volume of physiologic saline. At 1 h after treatment, three groups of mice treated with NAC and an equal number of saline controls were killed by cervical dislocation. The ascitic leukemia cells from each group were pooled after being harvested from the peritoneum and were then washed four times in iced physiological saline. The supernate from the final washing was saved, to be processed with the tumor cells. The cells in each group were counted in a hemocytometer after treatment with 0.1% trypan blue. An identical procedure was followed for animals killed 3 h after treatment with NAC or saline. 150 million viable cells from each experimental and control group were then processed exactly as described for the tissue homogenates except that after being shaken in the cold for 15 min, the tumor cells were sonically disrupted on ice for 45 s (three 15-s bursts, each separated by a 10-s cooling period) at a power output of 90 W using a Biosonik IV ultrasonicator (VWR Scientific, Inc., San Francisco, Calif.).

NPSH levels were determined from linear calibration curves using both reduced glutathione and NAC as standards in concentrations from 10 μ M to 1.5 mM; because results calculated from either curve were not statistically different, the data were expressed in terms of micromoles nonprotein sulfhydryl equivalents per gram wet weight for heart and liver, or per 1.5×10^8 cells for experiments with P388 leukemia. Since the intrinsic NPSH concentrations of both heart and liver each maintain a distinct pattern of diurnal variation (12), NPSH levels at every time point after NAC treatment have been compared to simultaneously-treated controls receiving physiologic saline. In preliminary experiments, we found that >90% of the NAC added as an internal standard to the tissue homogenate of a saline-treated control animal could be recovered as measurable nonprotein sulfhydryls. Three experimental samples and three control samples were each measured in duplicate for every time point investigated.

Glutamic-pyruvic transaminase determinations. The effect of NAC on the hepatotoxicity of doxorubicin was examined by determination of serum glutamic-pyruvic transaminase levels. It has recently been shown that treatment with doxorubicin in the dosage range used for our studies produces a significant rise in glutamic-pyruvic transaminase (GPT) levels 24 h after drug administration (22). Thus, we examined the effect of NAC on alterations in GPT levels produced by doxorubicin. 12 mice were divided into three, equal experimental groups. The first group was treated with physiologic saline (0.3 ml) *i.p.*; 1 h later these animals received a second, identical dose of saline. Group 2 was treated with an equal volume of saline but received doxorubicin (20 mg/kg *i.p.*) 1 h later. The final group of mice was treated with NAC (2,000 mg/kg *i.p.*) and then received 20 mg/kg doxorubicin *i.p.* in 1 h. 24 h after treatment with either saline or doxorubicin, the animals were killed by exsanguination through the retro-orbital plexus. Serum was separated by centrifugation; and GPT levels were determined spectrophotometrically at 25°C by a previously-described technique (23). GPT levels expressed as International Units per liter for this method were determined in duplicate for each sample in the three experimental groups.

Doxorubicin levels. To examine the effect of NAC administration on the pharmacokinetics of doxorubicin uptake in the heart, cardiac doxorubicin levels were measured in mouse heart by modification of the fluorometric method of Schwartz (24) as previously described (10). Eight experimental animals per time point were treated intraperitoneally with 15 mg/kg body wt of doxorubicin. Half of each group received 2,000 mg/kg NAC and half an equal volume of physiologic saline by gastric intubation 1 h before doxorubicin

injection. Eight control mice per time point were pretreated in a similar fashion and then received an equivolume dosage of saline intraperitoneally 1 h later.

Cardiac tissue was processed and drug-related fluorescence determined as previously reported (10). The relative fluorescent intensity of cardiac tissue homogenates, which reflects total doxorubicin content in heart (24), was corrected for fluorescent quenching by tissue pigments through the use of an internal doxorubicin standard added after homogenization to the saline-treated control hearts. In all determinations, background organ fluorescence, as determined in control animals, was converted to equivalent drug levels and subtracted from the experimental results.

Preliminary experiments showed that NAC at a concentration of 100 μ M (added directly to cardiac homogenates from saline-pretreated animals that had received doxorubicin) did not alter the measurement of drug-related fluorescence. Cardiac doxorubicin determinations for experimental samples were performed in triplicate for each time period sampled. The fluorescence measured in these experiments represents both unchanged doxorubicin and its fluorescent metabolites (25), and thus, drug levels have been expressed as nanogram doxorubicin equivalents per gram wet weight. Relative doxorubicin concentrations in animals pretreated with NAC have been compared to levels from simultaneously injected mice receiving saline pretreatment.

In order to study the effect of treatment with NAC on the metabolism of doxorubicin in the heart and liver, additional experiments were performed by a similar protocol but tissues were examined by a quantitative, fluorescent, thin-layer chromatographic technique that separates doxorubicin from its major metabolites in heart or liver (26). These studies were performed with an Aminco-Bowman spectrofluorimeter equipped with a thin-film scanner (Amino-Bowman, Inc., Silver Spring, Md.). Using this method, after chromatographic separation doxorubicin and doxorubicinol fluorescence is measured with respect to an internal daunorubicin standard to correct for tissue quenching and recovery. In these experiments, six experimental animals per time point received 15 mg/kg body wt of doxorubicin i.p. Half of each group received 2,000 mg/kg NAC and half physiologic saline by gastric intubation 1 h before doxorubicin injection. Six control mice per time point were pretreated similarly. An additional group of animals received NAC (2,000 mg/kg) i.p. before being treated with doxorubicin to investigate the effect of the route of NAC administration on doxorubicin accumulation. Cardiac and hepatic tissues were processed as previously described (10), and tissue metabolite levels were expressed as microgram drug equivalents per gram wet weight.

Morphological studies. Mice were divided into four experimental groups of five animals each as described for the animal studies above; half of all the animals received 2,000 mg/kg NAC by gastric tube; the other half were treated with equivalent volumes of saline. 1 h later, the mice received doxorubicin (20 mg/kg) or saline by intraperitoneal injection. 4 d after drug treatment, animals were killed by cervical dislocation. Hearts, livers, and gastrocnemius muscle were removed and vigorously washed free of erythrocytes in fixative. Areas from the left ventricle, left hepatic lobe, and left gastrocnemius muscle were cut into 1-mm slices and fixed by immersion in 2% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, for a total primary fixation time of 5 h at room temperature. The diced tissue was washed first in several rinses of 0.2 M sodium cacodylate buffer, pH 7.4, and then overnight in fresh buffer. The samples were postfixed in 2% aqueous osmium tetroxide for 2 h, dehydrated through a series of graded ethanol solutions,

transferred to propylene oxide, and embedded in Epon 812. Ultra-thin sections (60–100 μ m) were cut on a Porter-Blum MT-2B ultramicrotome, collected on 200 mesh uncoated copper grids, double stained with uranyl acetate and lead citrate, and examined with a Philips EM 201 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.). We also examined the effect of NAC on cardiac morphology after doxorubicin treatment in animals carrying P388 leukemia. These animals were implanted i.p. with one million P388 cells; one day later they were treated in a fashion similar to the non-tumor-bearing mice.

To evaluate quantitatively the effect of NAC on doxorubicin cardiac toxicity, representative electron micrographs were printed from each sample and the magnitude of cytopathologic changes in fine structure were determined blindly using the 0–3 scale described by Bristow et al. (2). A score of 0 indicated no change from normal; a score of 3 was used for sections showing diffuse myocyte damage, vacuolization, and frank necrosis. The highest score for each sample was used as an index of the severity of cardiac damage. Scores were tabulated for each experimental group and the mean and standard error calculated.

For light microscopic studies, samples of mouse jejunum obtained after following a protocol identical to that for heart, liver, and skeletal muscle were perfused *in situ* and then fixed in 10% buffered formalin. Sections were embedded in paraffin and stained with hematoxylin and eosin. Light micrographs were printed and examined for the morphological alterations in the intestine that have been previously demonstrated to be associated with doxorubicin treatment (27).

Statistical analyses. Data were analyzed with the two-tailed Student's *t* test for independent means (NS, $P > 0.05$ [28]). Survival data were compared by the log rank test (29), and a computation of the beta error for the experimental sample sizes used in these studies was determined using standard statistical sources (30, 31). Data are expressed as the mean \pm 1 SE.

RESULTS

Effect of NAC on survival following a single dose of doxorubicin. To examine the hypothesis that enhancement of cardiac sulfhydryl group content might ameliorate doxorubicin toxicity in mice, we initially chose to pretreat experimental animals before doxorubicin administration with pharmacologic dosages of NAC, an agent that had been shown by others to increase the nonprotein sulfhydryl level of rat spleen (32), and mouse kidney, and lung (33). In order to provide an adequate test of NAC as a protective agent, we used a dose of doxorubicin (20 mg/kg i.p.) that our previous studies had suggested would be almost uniformly lethal (10) and would produce definite histological evidence of a drug-induced cardiomyopathy (4).

Treatment with NAC, in a dose of 2,000 mg/kg given 1 h before the injection of doxorubicin, significantly improved both short- and long-term animal survival (Table I). At the end of the 56-d observation period, the median survival for mice receiving the highest dose of NAC had not been reached; furthermore, unlike the saline-pretreated control group, no experimental animals receiving NAC died more than 25 d after doxorubicin was administered. As shown in Table I,

TABLE I
Effect of NAC on Survival After Doxorubicin Treatment*

NAC dose	Timing of NAC dose with respect to doxorubicin administration			
	3 h before	1 h before	Simultaneous	1 h after
mg/kg				
0†	6.67% (n = 30, 7 d)	0% (n = 44, 5 d)	0% (n = 10, 5 d)	6.25% (n = 16, 6 d)
100		25% (n = 20, 7 d)§		
750		30% (n = 20, 8 d)§		
2000	36% (n = 25, 7 d)¶	62.3% (n = 53, > 56 d)§¶**	21.7% (n = 23, 5 d)	22.7% (n = 22, 6 d)

* Doxorubicin was administered to all mice in a dose of 20 mg/kg by intraperitoneal injection; NAC was given by gastric intubation in the dose and at the times indicated. Data are expressed as percent survival 56 d after doxorubicin treatment; the total number of mice per experimental group (n) and the median survival figure in days are given in parentheses.

† Control animals received equivolume dosages of physiologic saline instead of NAC at the times indicated. No significant differences between survivals of any group receiving saline rather than NAC.

§ Significantly different from saline-pretreated control group ($P < 0.001$).

¶ Significantly different from saline-pretreated control group ($P < 0.01$).

¶ Significantly different from groups pretreated at the same time with lower doses of NAC ($P < 0.025$).

** Significantly different from group treated 3 h before ($P < 0.05$), simultaneous with ($P < 0.005$), or 1 h after doxorubicin ($P < 0.005$).

the protective effect of NAC depended upon the dose of drug administered; though pretreatment with either 100 or 750 mg/kg NAC significantly prolonged survival compared to controls receiving physiological saline ($P < 0.01$), the highest dose of NAC used (2,000 mg/kg) prolonged survival to a significantly greater degree than both lower NAC dosages ($P < 0.025$). The timing of NAC treatment was also critically important in determining its relative protective efficacy. NAC was not significantly effective if administered simultaneously with or 1 h after doxorubicin; pretreatment 3 h, rather than 1 h, before doxorubicin also diminished ($P < 0.05$), but did not abolish ($P < 0.01$), the survival benefit of therapy with NAC (Table I).

Though the dose and timing of treatment with NAC significantly affected animal survival after doxorubicin, the route of NAC administration did not. NAC given intraperitoneally in a dose of 2,000 mg/kg 1 h before doxorubicin (20 mg/kg i.p.) resulted in a 52.2% survival at 56 d after drug treatment ($n = 23$) compared to a 0% ($n = 12$, 4 d median) survival for saline-pretreatment i.p. ($P < 0.01$). The survival advantage for intraperitoneal NAC was not statistically different from NAC administered by gastric intubation.

NAC itself, whether given by the intraperitoneal ($n = 11$) or gastric ($n = 20$) route at a dose of 2,000 mg/kg produced no mortality during the 8 wk of experimental observation; similar results were observed for animals receiving saline alone.

Effect of NAC on the toxicity of multiple doses of doxorubicin. Because NAC provided significant survival benefit to animals treated with a single large dose of doxorubicin, we examined its protective activity in

mice treated with multiple drug doses, a schedule that more closely approximates the administration of doxorubicin in man (34).

NAC pretreatment significantly improved long-term survival after repeated, small doses of doxorubicin. 10 wk after the final doxorubicin injection, survival in the group treated with NAC was 51.4% ($n = 35$) compared to 16.7% ($n = 30$) for animals receiving saline before doxorubicin ($P < 0.01$). At both 4 ($P < 0.05$) and 7 ($P < 0.001$) wk from the start of doxorubicin treatment, NAC administration also provided a survival advantage; 91.4 vs. 73.3% survival for the former groups, 80.0 vs. 23.3% for the latter. Repeated intraperitoneal dosages of NAC ($n = 10$) or saline ($n = 5$) to animals not receiving doxorubicin resulted in no observed mortality. Furthermore, doxorubicin administration significantly decreased mouse heart and body weight at the end of the observation period (Table II). Animals pretreated with NAC, on the other hand, had a significantly smaller decrease in both total body weight ($P < 0.05$) and heart wet weight ($P < 0.02$) compared to doxorubicin-treated animals receiving saline. However, heart weight to body weight ratios in all four experimental groups were not statistically different; since heart weight and total body weight are intimately linked in most species (35), the protective action of NAC on heart wet weight might be a nonspecific effect, reflecting decreased systemic toxicity from doxorubicin rather than a primary cardio-sparing activity.

Effect of NAC on the histology of doxorubicin toxicity in the heart, liver, skeletal muscle, and small intestine. Doxorubicin produces a characteristic pat-

TABLE II
Effect of NAC on Changes in Heart and Body Weight Produced
by Multiple Doses of Doxorubicin*

Experimental group	Body weight† g	Heart weight† mg	Heart weight Body weight (× 10 ³)‡
Saline control (n = 5)	31.02±0.36	159.2±3.6	5.13±0.15
NAC control (n = 10)	30.44±0.39	154.4±4.68	5.07±0.16
Doxorubicin and saline (n = 5)	21.36±0.54§	102.8±1.53§	4.81±0.07
Doxorubicin and NAC (n = 18)	25.43±1.41§¶	123.0±4.17§¶	4.84±0.16

* Doxorubicin (5 mg/kg) or saline was administered i.p. every 2 wk for a total of three doses; 1 h before each treatment, NAC (2,000 mg/kg) or saline was injected i.p. 10 wk after the last doxorubicin injection, animals were weighed and then killed by cervical dislocation; hearts were removed, washed in saline, blotted dry, and the wet weight determined.

† Data for body weight, heart weight, and heart weight/body weight ratio expressed as the mean±1 SE.

§ Significantly different from both saline and NAC control groups ($P < 0.01$).

¶ Significantly higher than saline-pretreated animals receiving doxorubicin ($P < 0.05$).

¶ Significantly higher than saline-pretreated animals receiving doxorubicin ($P < 0.02$).

tern of morphological abnormalities in a variety of different tissues (9, 27). In particular, several different species, including the mouse, develop a drug-induced cardiomyopathy that has histological features similar to the cardiomyopathy produced by doxorubicin in man (4, 9). In this study, we investigated the effect of NAC pretreatment on doxorubicin-induced injury to heart, liver, skeletal muscle, and jejunal mucosa. Tissues were sampled 4 d after doxorubicin treatment because our preliminary results suggested that morphological abnormalities were maximal at this time point. Fig. 1A

is a section of left ventricular myocardium from an animal in the saline control group. The myofibrils are compact, regularly aligned, and are interspersed by rows of mitochondria of normal appearance. Hearts from animals in the NAC control group were also of normal appearance (data not shown). Fig. 1B is an example of a myocardial section from a doxorubicin-treated animal that had received physiological saline 1 h before drug administration. This figure, which is typical of the type of injury that occurred in the entire experimental group, demonstrates the marked degree

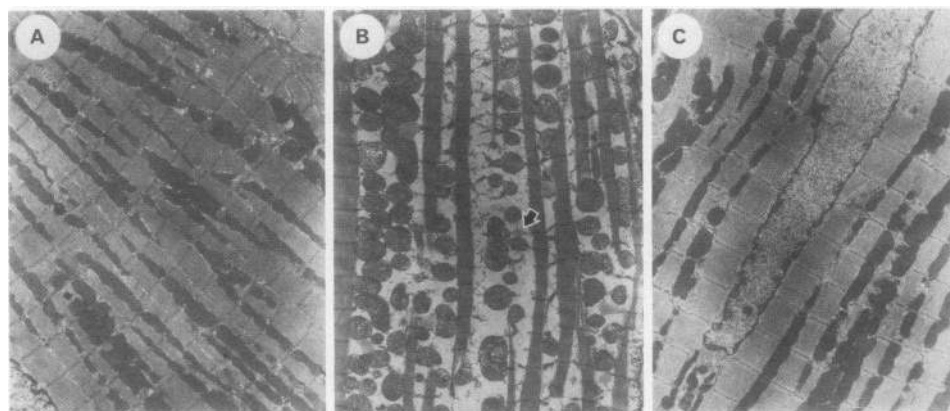


FIGURE 1 Effect of NAC on doxorubicin-induced damage to heart muscle. (A) Electron micrograph of left ventricular myocardium taken from an animal 96 h after treatment with physiological saline. Myofibrillar and mitochondrial architecture are intact. $\times 11,700$. (B) Left ventricular section 96 h after doxorubicin (20 mg/kg i.p.). Animals in this group were pretreated with saline by gastric intubation. Cytoplasmic and mitochondrial edema are marked; myocytolysis has begun, sarco-plasmic reticulum is disrupted (arrow), and mitochondrial cristae are disorganized. $\times 11,700$. (C) Left ventricular myocardium 96 h after the same dose of doxorubicin as in B. Animals from this experimental group received NAC (2,000 mg/kg) by gastric tube 1 h before doxorubicin. Features of doxorubicin cardiomyopathy are absent and morphology closely resembles saline controls. $\times 11,800$.

of myofibrillar disruption and lysis of myofilaments that may be produced by doxorubicin. There is extensive interstitial edema with alterations in Z-band registry; the mitochondria are swollen with disorganization of the cristae. Vacuolated elements of sarcoplasmic reticulum are also seen. In contrast, myocardial architecture in the group pretreated with NAC (Fig. 1C) is intact and closely resembles control samples from mice treated with saline (Fig. 1A).

To quantitate the protective effect of NAC, multiple coded sections from animals in each of the four experimental groups were scored on a scale from 0 to 3 for the distinctive features of doxorubicin cardiac toxicity. Ultrastructural changes were not found in the saline- and NAC-control groups (pathologic scores for each 0.00 ± 0.00 ; mean \pm SE). Mice receiving doxorubicin that had been pretreated with saline had a mean pathologic score of 2.75 ± 0.25 , reflecting severe morphological abnormalities. On the other hand, animals treated with NAC before doxorubicin administration had a cardiac pathologic score of 0.25 ± 0.25 , which was significantly less than the score for saline-pretreated mice, $P < 0.01$. In mice bearing P388 leukemia, similar morphological observations were made.

Doxorubicin also damaged the liver (data not shown). The livers of doxorubicin-treated mice showed cytoplasmic attenuation and swelling of the mitochondria and endoplasmic reticulum; mice pretreated with NAC developed a similar pattern of injury. Furthermore, the livers of NAC-pretreated animals not receiving doxorubicin also showed evidence of lipid droplet accumulation and cytoplasmic attenuation (data not shown) suggesting that NAC itself, in pharmacologic dosages, may produce reversible hepatotoxicity.

Mice treated with doxorubicin also develop mucosal damage to the small intestine. Thus, the survival advantage of doxorubicin-treated mice receiving NAC could be related to an amelioration of gastrointestinal toxicity. We found that doxorubicin produced shortening and ulceration of the jejunal mucosa. However, jejunal mucosa from animals pretreated with NAC was also abnormal; villi remained atrophic and mucosal ulceration was present and was comparable to that seen in saline-pretreated mice. Thus, weight loss in both groups of mice treated with doxorubicin could be due to malabsorption resulting from the effect of the drug on the small intestinal mucosa.

Unlike heart, liver, and jejunal mucosa, we did not observe any ultrastructural alterations by light or electron microscopy in mouse gastrocnemius muscle after the intraperitoneal administration of doxorubicin. Thus, no statement can be made regarding the effect of NAC on this tissue. This lack of ultrastructural change may be related to the low level of doxorubicin that is found in skeletal muscle after intraperitoneal drug treatment (36).

From these studies, it would appear that the toxicity of doxorubicin for rapidly proliferating tissues, such as small intestinal mucosa, is not substantially prevented by NAC. This may explain why NAC-pretreatment does not provide complete protection for mice receiving doxorubicin. In contrast, the architecture of heart muscle is readily preserved by NAC; this finding adds specificity to the survival studies reported above and may underlie the long-term survival advantage of NAC-pretreated animals.

Effect of NAC on glutamic-pyruvic transaminase levels after doxorubicin treatment. To expand our morphological observation that NAC did not prevent doxorubicin hepatotoxicity, we examined the effect of thiol pretreatment on the elevation of serum GPT level which follows treatment with doxorubicin in the mouse (22). 24 h after receiving physiological saline, the serum GPT level in our experimental animals was 11.73 ± 0.82 IU/liter (mean \pm 1 SE). In the experimental group receiving doxorubicin (20 mg/kg i.p.) 1 h after saline pretreatment, the GPT level was significantly elevated, 54.65 ± 4.72 IU/liter, compared to the saline control group, $P < 0.01$. The GPT activity of animals treated with NAC before doxorubicin was also significantly different from that in the saline-control group, 30.45 ± 5.44 IU/liter, $P < 0.05$. However, the GPT levels of the two groups treated with doxorubicin were also significantly different, $P < 0.02$. Thus, whereas morphological evidence for a protective effect of NAC on doxorubicin hepatotoxicity is lacking, the GPT data indicate that animals pretreated with NAC may be spared some degree of hepatic injury after doxorubicin administration.

Effect of NAC on the chemotherapeutic activity of doxorubicin. After investigating the protective effect of NAC on doxorubicin cardiac toxicity, we examined the relationship between NAC administration and the chemotherapeutic activity of doxorubicin. In these experiments, we studied animals implanted with P388 leukemia, an experimental murine tumor that in its ascitic form is sensitive to intraperitoneal treatment with doxorubicin (37). For this investigation, tumor cells, doxorubicin, NAC, and saline were all present intraperitoneally to maximize the potential for any drug-drug interaction. Fig. 2 shows that P388 ascites tumor is rapidly fatal in mice treated with either saline ($n = 30$) or NAC alone ($n = 30$); median survival time was 8 d in both groups, with no long-term survivors in either group and no statistical difference between treatment arms. Animals receiving doxorubicin (10 mg/kg i.p.) after saline-pretreatment ($n = 57$) had a 20-d median survival with 24.6% (14 of 57) of the group alive at 56 d; this survival pattern was statistically superior ($P < 0.001$) to either saline- or NAC-treated controls. Tumor-bearing animals pretreated with NAC before receiving doxorubicin ($n = 57$) had a 25-d median life-

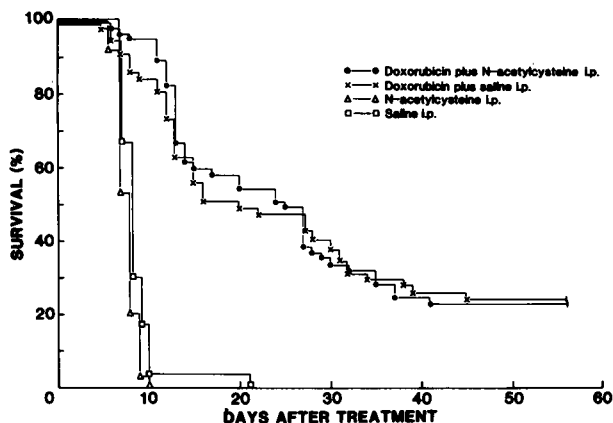


FIGURE 2 Effect of NAC on the response of P388 ascites tumor to doxorubicin (10 mg/kg i.p.). Mice were implanted intraperitoneally with one million P388 leukemia cells; 24 h after implantation the animals were treated with NAC (2,000 mg/kg) or an equal volume of saline i.p. 1 h following pretreatment, doxorubicin (10 mg/kg) or saline was administered by intraperitoneal injection. Survival was followed for 56 d after treatment. $n = 30$ for both the saline and NAC control groups; $n = 57$ for each group treated with doxorubicin.

span, with 22.8% (13 of 57) long-term survivors; the survival pattern of these animals was also superior ($P < 0.001$) to both saline- and NAC-treated controls. By a routine test of significance (the log rank test), the survival of animals pretreated with NAC was not different from that of mice receiving the same dose of doxorubicin but pretreated with saline. Furthermore, at a significance level of 5% and with a power (1-beta) of 90%, for the sample sizes used in this experiment there could be no more than a 20% difference in survival between the saline- and the NAC-pretreated groups receiving doxorubicin (30).

Similar results were obtained using a lower dose of doxorubicin. Mice administered 7.5 mg/kg doxorubicin i.p. after NAC pretreatment ($n = 30$) had a 13-d median survival with 6.7% (2 of 30) of the group alive at 56 d. This was significantly better than either control group, $P < 0.001$. Animals pretreated with saline ($n = 30$) also had a 13-d median survival rate with 3.3% (1 of 30) long-term survivors, which was also significantly longer than both control groups, $P < 0.001$. The survival patterns of the saline- and NAC-pretreated groups receiving doxorubicin were not significantly different by the log rank test. Furthermore, the sample sizes used in this experiment indicate that with a power of 80% at the 5% significance level there would at most be no more than a 30% difference in survival between the two experimental arms.

These results suggest that a dose of NAC (2,000 mg/kg) that ablates electron microscopic evidence of doxorubicin cardiac toxicity does not interfere with the drug's antitumor activity against P388 leukemia.

Effect of NAC on cardiac, hepatic, and tumor cell nonprotein sulfhydryl levels. In an attempt to explain the enhanced survival of doxorubicin-treated mice that had received NAC, we examined the effect of NAC on the nonprotein sulfhydryl group content of heart and liver. Fig. 3 shows that cardiac NPSH levels rose rapidly after NAC was administered by intraperitoneal injection. NPSH levels in heart 30 min ($317 \pm 35\%$; percent control ± 1 SE) and 60 min ($429 \pm 60\%$) after 2,000 mg/kg NAC were significantly higher ($P < 0.01$) than levels in control animals simultaneously treated with physiological saline. Cardiac nonprotein sulfhydryl group content declined rapidly thereafter; and was not significantly different from control 90 min ($132 \pm 8\%$), 120 ($107 \pm 12\%$), and 180 ($105 \pm 10\%$) min after injection. At the same dose of NAC, hepatic NPSH content at 30 ($96 \pm 20\%$), 60 ($109 \pm 4\%$), 120 ($76 \pm 6\%$), and 180 ($82 \pm 9\%$) min after treatment did not differ from control levels; 90 min after NAC adminis-

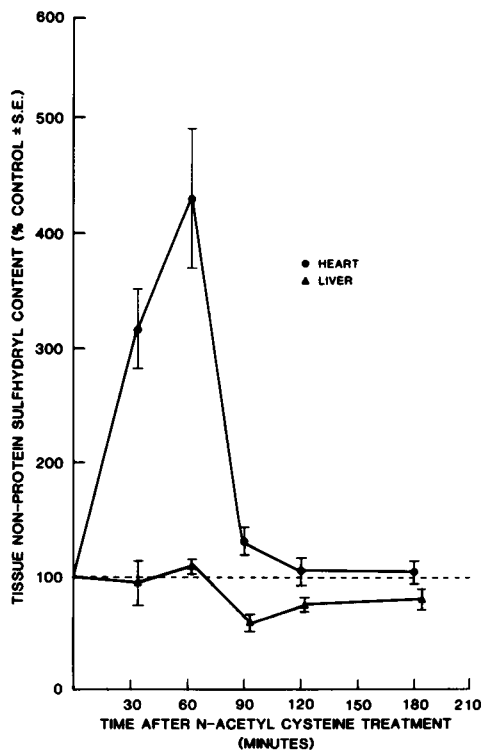


FIGURE 3 Effect of NAC on cardiac and hepatic nonprotein sulfhydryl content. Mice were treated with NAC (2,000 mg/kg i.p.) or an equivolume dose of saline; nonprotein sulfhydryl concentrations were determined in hearts and livers from NAC-treated animals and compared to those from control animals simultaneously treated with physiologic saline. 1 h after injection, cardiac nonprotein sulfhydryl content was (mean ± 1 SEM) $5.53 \pm 0.78 \mu\text{mol/g}$ in the NAC group compared to $1.28 \pm 0.14 \mu\text{mol/g}$ in the saline-controls ($P < 0.01$). At the same time, hepatic nonprotein sulfhydryl levels in animals receiving NAC ($7.75 \pm 0.27 \mu\text{mol/g}$) were equivalent to those receiving saline ($7.07 \pm 0.37 \mu\text{mol/g}$) (NS).

tration hepatic NPSH content was significantly lower ($60 \pm 8\%$, $P < 0.05$) than in corresponding control animals.

The effect of NAC on the NPSH concentration of ascitic P388 leukemia cells was also examined. 1 h after treatment with NAC (2,000 mg/kg i.p.), tumor cell NPSH content (mean \pm 1 SE; $1.62 \pm 0.14 \mu\text{mol}/1.5 \times 10^8$ cells) was nearly three times higher than that in the control animals treated simultaneously with physiological saline ($0.59 \pm 0.02 \mu\text{mol}/1.5 \times 10^8$ cells), $P < 0.02$. 3 h after thiol administration the NPSH level in P388 cells from animals receiving NAC ($0.67 \pm 0.03 \mu\text{mol}/1.5 \times 10^8$ cells) was equivalent to that in the saline control group ($0.70 \pm 0.02 \mu\text{mol}/1.5 \times 10^8$ cells), NS. The physiological saline used for the final washing of the P388 cells processed 1 h after NAC administration contained no detectable NPSH level by the methods used in these experiments, suggesting that the NPSH concentrations measured in the P388 tumor reflect intracellular thiol content.

The administration of NAC produces remarkably different effects on cardiac and hepatic NPSH pools, which may help to explain why NAC pretreatment substantially ameliorates the toxicity of doxorubicin on the heart rather than the liver. Furthermore, the pharmacokinetic profile of NAC uptake in P388 cells seems to parallel that for cardiac tissue. Although for technical reasons the experiments with P388 leukemia were performed on animals after 6 d, rather than one day, of tumor growth and therefore are not strictly comparable to our survival studies of P388-bearing mice treated with doxorubicin, this investigation does suggest that enhancement of the NPSH content of both mouse heart and P388 cells by NAC leads to substantially different effects on the cytotoxicity of doxorubicin in the two tissues.

Effect of NAC on the pharmacokinetics of doxorubicin uptake in the heart and liver. To determine whether the protective effect of NAC could be explained by an altered pattern of cardiac or hepatic doxorubicin accumulation, we examined the pharmacokinetics of doxorubicin uptake in the heart and liver after pretreatment with NAC. As shown in Table III, doxorubicin reaches the heart rapidly after intraperitoneal administration, attaining peak concentration 1–3 h after injection. Animals pretreated with NAC before doxorubicin do not have a statistically different pharmacokinetic time-course compared to mice pretreated with saline (Table III). By a different experimental technique that extracts doxorubicin from tissues more effectively, we also found that the administration of NAC either intraperitoneally or by gastric intubation does not affect the concentration of doxorubicin in the heart or liver 1 h after drug treatment (Table IV). Furthermore, 24 h after doxorubicin administration, levels of doxorubicin, doxorubicinol,

TABLE III
Effect of NAC on Doxorubicin Uptake into the Heart*

Hours after doxorubicin	Cardiac doxorubicin concentration	
	Saline pretreatment	NAC pretreatment
	ng doxorubicin equivalents/g†	
0.5	2,255 \pm 144	2,452 \pm 607
1	2,396 \pm 284	2,766 \pm 362
3	2,776 \pm 87	2,332 \pm 369
18	1,459 \pm 35	1,681 \pm 87
24	1,391 \pm 49	1,174 \pm 69
48	823 \pm 113	801 \pm 51

* Experimental animals were pretreated with NAC (2,000 mg/kg) or saline by gastric intubation. 1 h later they received doxorubicin 15 mg/kg i.p. Mice were sacrificed at the times indicated and hearts processed for determination of doxorubicin concentration. Data represent mean \pm 1 SE of triplicate determinations on four samples per time point for both saline and NAC pretreatment groups.

† No significant differences between pretreatment groups at any time point sampled.

and an unidentified doxorubicin polar metabolite in both heart and liver were not significantly different for animals receiving saline or NAC pretreatment (Table IV). Thus, it appears that the survival advantage of doxorubicin-treated mice receiving NAC is not conferred solely by a change in the accumulation of doxorubicin or its metabolites in the heart or liver.

DISCUSSION

There is an increasing body of experimental evidence suggesting that the life-threatening effects of doxorubicin on the heart result from drug-induced, oxidative tissue injury (5, 7). In particular, our previous studies indicated that doxorubicin-related reactive oxygen metabolism may overwhelm the limited capacity of cardiac muscle to detoxify free radicals, resulting in extensive peroxidative damage to myocardial cells (4, 10). In the present study, we have demonstrated histologically that treatment of experimental animals with pharmacologic dosages of the sulfhydryl *n*-acetylcysteine selectively rescues the heart, and not the liver or small intestine, from the toxicity of doxorubicin. This selective, cardioprotective action may explain why NAC did not completely abolish drug-related mortality in these experiments.

NAC might also have improved survival after doxorubicin treatment by limiting drug-induced leukopenia and thrombocytopenia; however, such an effect was not investigated in these studies because previous experiments had shown that the hematological toxicity of doxorubicin in the species of mice used for our

TABLE IV
Effect of NAC on Doxorubicin Metabolism in Heart and Liver*

Hours after doxorubicin	Pretreatment and route of its administration	Heart†			Liver‡		
		D	D-ol	P	D	D-ol	P
		µg drug equivalent/g§			µg drug equivalent/g§		
1	Saline (p.o.)	18.25±2.03			42.32±3.39		
	NAC (p.o.)	22.53±6.16			48.62±0.97		
	NAC (i.p.)	14.96±4.06			36.05±4.02		
24	Saline (p.o.)	10.25±1.17	4.11±0.01	7.58±0.75	10.65±1.07	1.63±0.57	1.12±0.56
	NAC (p.o.)	12.54±0.67	4.14±0.16	6.28±0.59	9.61±2.65	2.53±0.34	1.21±0.21

* Experimental design the same as that described in the legend to Table III, except that hearts and livers were processed for doxorubicin metabolites by a different technique as indicated in Methods. Data represent the mean ± 1 SE of duplicate determinations on three samples from each experimental group.

† Concentrations of doxorubicin and its metabolites in heart and liver are expressed as drug equivalents/gram wet weight of D (parent doxorubicin), D-ol (doxorubicinol), and P (an unidentified, polar doxorubicin metabolite).

§ No significant differences between the concentrations of doxorubicin or its metabolites in heart or liver in any pretreatment group, regardless of the route of administration.

investigations is mild, and probably contributes little to drug-related morbidity (38).

NAC treatment produced more than a fourfold increase in cardiac nonprotein sulfhydryl content. When doxorubicin was administered coincident with this peak cardiac NPSH level, the protective activity of NAC on the heart was maximized. Previous investigators have demonstrated a similar time-course in other organs for the increase in NPSH concentration following NAC-injection (33, 39); furthermore, in those studies, it is apparent that the enhancement of tissue NPSH levels is dose-dependent, and can rarely be measured below a dose of 100 mg/kg NAC. The dose-related effect of NAC on tissue NPSH content may explain why NAC provided a lesser survival advantage for doxorubicin-treated mice when administered in a dosage of 750 or 100 mg/kg.

After oral or intraperitoneal injection, NAC is at least partially deacetylated to l-cysteine in the liver (39); experiments by Ball (32) in the rat have previously demonstrated that the NPSH content of several organs increases after l-cysteine administration and that this increase is related to the concentration of l-cysteine in tissue rather than to enhanced GSH synthesis. If these results apply to mouse heart, they suggest that NAC could have a direct cardioprotective effect in doxorubicin-treated animals that may be separable from its contribution to the gamma-glutamyl cycle.

We have also shown that NAC-pretreatment does not affect the pharmacokinetic profile or metabolism of doxorubicin in the heart or liver; this seems to exclude a change in the tissue uptake or handling of doxorubicin as an explanation for our results. Furthermore, it has recently been shown that the same flavin-containing

enzymes that metabolize doxorubicin under anaerobic conditions, produce the semiquinone free radical intermediate in the presence of molecular oxygen (6). If NAC were to alter the metabolic degradation of doxorubicin, it should also interfere with the conversion of doxorubicin to its free radical by these enzyme systems; recent studies in our laboratory suggest that this is not the case (unpublished observations).

Though doxorubicin is converted to its free radical-metabolite by microsomes from several different experimental tumor cells (5), NAC did not appear to interfere with the drug's chemotherapeutic effect in P388 leukemia. For the sample size chosen in our survival study using 10 mg/kg doxorubicin, the probability that the true response rates of P388-bearing animals receiving chemotherapy with either NAC or saline pretreatment actually differed by 20%, that is the probability of such a false negative or Type II error in our study, was 10%, a generally acceptable limit for the Type II error in clinical investigations (40). On the other hand, in these experiments a substantial increase in the therapeutic efficacy of doxorubicin against P388 leukemia after NAC pretreatment could have been difficult to observe because NAC does not markedly diminish either the intestinal or hepatic toxicity of doxorubicin. Thus, the cardioprotective effect of thiol administration may be more apparent as a survival advantage in normal animals than in the small group of tumor-bearing mice that are cured by a single-dose schedule of doxorubicin treatment (37). Freeman et al. (41) have recently found that NAC does not decrease the therapeutic activity of doxorubicin against the Erlich ascites carcinoma; when doxorubicin was administered using a multiple, low-dose treatment

schedule in their study, NAC significantly enhanced the chemotherapeutic efficacy of doxorubicin. These results suggest that a major cytotoxic effect of doxorubicin on tumor cells may not be related to free radical formation; in that circumstance, NAC could have its predominant impact on the cardiac damage produced by doxorubicin rather than its antineoplastic activity.

There are several possible mechanisms for the cardioprotective action of NAC that we observed in doxorubicin-treated mice. NAC is a potent sulfhydryl agent that could reduce lipid peroxides formed *in vivo* after doxorubicin administration (4) to nontoxic sulfhydryl conjugates (18, 19); or, in the aqueous compartment it might prevent the initiation of lipid membrane peroxidation. NAC is also an avid hydroxyl radical scavenger ($k[\text{mol}^{-1}\text{s}^{-1}] = 8 \times 10^9$ [17]) and radio-protectant that can break free radical chain reactions without itself producing damaging, sulfur-containing radical by-products (17). This may be especially important in view of the potential for doxorubicin-induced hydrogen peroxide accumulation in the myocardial cell (7). NAC could also help to maintain essential cardiac membrane sulfhydryls and sulfhydryl-containing enzymes (such as glyceraldehyde-3-phosphate dehydrogenase) in the reduced state, either directly by disulfide interchange (42, 43) or through its oxygen radical scavenging activity (17). Cardiac energy metabolism and membrane integrity may be preserved in this fashion.

NAC may specifically protect the heart against doxorubicin because the turnover of reduced glutathione in this organ is so slow, especially when compared to kidney or liver (44). Hence, in addition to its limited intrinsic enzymatic defenses against oxygen radicals, the reduced sulfhydryl pool in the heart after doxorubicin treatment may be insufficient to prevent the oxidation of critical intracellular constituents. The administration of NAC provides a temporary alternate source of cardiac reducing equivalents that, in the appropriate concentration (17), may limit the adverse effects of a doxorubicin-induced free radical cascade.

If further studies confirm the differential impact of drug-related oxygen radical metabolism on heart cells and human tumor, sulfhydryl treatment with NAC could be used in controlled attempts to improve the therapeutic index of doxorubicin.

ACKNOWLEDGMENTS

We wish to thank Ms. Jill Reeves for her excellent technical assistance and Dr. Ken Chan, University of Southern California School of Pharmacy, for his help in the determination of doxorubicin metabolite levels. This work was supported by grant 622-G2-2 from the American Heart Association-Greater Los Angeles affiliate, and, in part, by P. H. Service grant

29463-01 awarded by the National Cancer Institute, Department of Health and Human Services. Dr. Doroshow is a Special Fellow of the Leukemia Society of America.

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