Adriamycin and Daunomycin Generate Reactive Oxygen Compounds in Erythrocytes

Adriamycin and daunomycin produce dose-related cardiac toxicity that may be related to oxygen radicals. Addition of these compounds to human erythrocyte suspensions resulted in stimulation of hexose monophosphate shunt activity that was markedly impaired in the absence of oxyhemoglobin. Evidence for generation of hydrogen peroxide by these compounds was provided by oxidation of reduced glutathione, by $^{14}$C-formate oxidation, and by the catalase-aminotriazole trapping technique. These experiments indicate that Adriamycin and daunomycin interact with oxyhemoglobin to generate reactive oxygen metabolites. A similar interaction with oxymyoglobin may occur in the heart and produce oxygen radicals that injure cardiac myocytes.

Adriamycin (doxorubicin HCl) and its closely related anthracycline analogue daunomycin are important agents in cancer chemotherapy. The use of these drugs, however, is limited by a dose-related cardiomyopathy. Several lines of evidence suggest that this cardiac toxicity may be related to generation of oxygen radicals and lipid peroxidation. Anthracyclines contain quinone structures that potentially may be reduced in vivo and then auto-oxidize to produce hydrogen peroxide and possibly other reactive oxygen metabolites. Incubation of Adriamycin and daunomycin with microsomal fractions, or with purified cytochrome p450 reductase and NADPH, results in the generation of superoxide and hydrogen peroxide and produces lipid peroxidation. Pretreatment with the antioxidant tocopherol before administration of Adriamycin to mice bearing ascites tumors markedly reduces lipid peroxidation in the heart and ameliorates cardiotoxicity without apparently impairing tumor response. Other data suggest that myocardial glutathione and glutathione peroxidase are important in protecting against Adriamycin-induced cardiac injury in mice, presumably by scavenging oxygen radicals. However, despite this suggestive evidence, definite generation of reactive oxygen species in intact cells has not been demonstrated.

Since the anthracyclines seemed likely to generate reactive oxygen compounds in other tissues besides myocardium, we wanted to determine if generation of oxygen radicals by Adriamycin and daunomycin could be detected in...
intact human erythrocytes. Erythrocytes were studied because they are easily obtained and their metabolic handling of oxygen metabolites is well described.\textsuperscript{10,11} The primary mechanism for protecting against injury by hydrogen peroxide and lipid hydroperoxides in erythrocytes is reduction of these compounds by reduced glutathione (GSH), catalyzed by glutathione peroxidase.\textsuperscript{10} GSH is then regenerated by the enzyme glutathione reductase with simultaneous oxidation of NADPH. NADPH is subsequently regenerated by the oxidative portion of the hexose monophosphate shunt (HMPS). Thus generation of oxygen metabolites causes stimulation of HMPS activity by these linked metabolic reactions.\textsuperscript{10,11} Several drugs have been found to produce stimulation of the HMPS by acting as intermediates in the transfer of electrons from NADPH to oxyhemoglobin or other suitable electron acceptors; these include methylene blue\textsuperscript{12} and primaquine and phenylhydrazine.\textsuperscript{4}

**MATERIALS AND METHODS**

*Preparation of erythrocytes.* Venous blood was collected in heparinized tubes from normal volunteers and was centrifuged at 1000 g for 10 min. The plasma and buffy coat were removed and the RBC were washed once in 5 vol saline and resuspended in buffer pH 7.4 containing 145 mM sodium, 5 mM potassium, 20 mM glycylglycine, 5 mM glucose, and 145 mM chloride. As reported previously, pH values in this incubation system were maintained at 7.3 or greater for more than 3 hr.\textsuperscript{12,13} Leukocyte and platelet contamination were less than 1000/mm\textsuperscript{3} and 10,000/mm\textsuperscript{3}, respectively.

*Metabolic studies.* Packed RBC (3–4 ml) were resuspended in buffer to a final volume of 10 ml in a 50-ml triple-headed distilling flask to which was then added 5 µCi radioactive substrate. All radioactive reagents were obtained from Amersham/Searle, Arlington Heights, Ill. The inlet of the flask was connected to a gas cylinder containing either compressed air, carbon monoxide (CO), or 6\textsuperscript{14}CO\textsubscript{2} in air. The last gas mixture produces nearly 100\textsuperscript{a}, carboxyhemoglobin while maintaining a physiologic partial pressure of oxygen (approximately 140 mm Hg). A second arm of the flask was covered with a rubber stopper through which reagents could be added or samples withdrawn with a spinal needle. The outlet arm of the flask was connected to a Cary-Tolbert ionization chamber electrometer apparatus (Cary Instruments, Monrovia, Calif.). The use of this apparatus for continuous monitoring of \textsuperscript{14}CO\textsubscript{2} produced by cell suspensions have been described in detail elsewhere.\textsuperscript{12,13} A duplicate system was used so that \textsuperscript{14}CO\textsubscript{2} derived from glucose-1\textsuperscript{14}C could be measured simultaneously from both control and experimental flasks. The incubation flasks were maintained at 37°C throughout the experiments and were stirred continuously. After baseline \textsuperscript{14}CO\textsubscript{2} production was established, agents (in buffer) were added through the center well to the experimental flasks and an equal volume of buffer alone to the control flasks. \textsuperscript{14}CO\textsubscript{2} production was calculated from the millivolt reading once steady-state conditions were reestablished in the experimental chambers and compared to the corresponding values from the control flasks. \textsuperscript{14}CO\textsubscript{2} production was calculated as previously described and expressed as µmol CO\textsubscript{2}/ml RBC/hr.\textsuperscript{13}

**Studies to Detect Peroxide Generation**

Three methods were used to detect generation of hydrogen peroxide in RBC incubated with Adriamycin or daunomycin.

*Glutathione stability.* RBC were washed thoroughly and resuspended in glycylglycine buffer with or without glucose and with Adriamycin or daunomycin. (Adriamycin HCl was provided by Adria Laboratories and daunorubicin HCl by the Drug Development Branch of the National Cancer Institute.) RBC-reduced glutathione (GSH) concentrations were then determined before and after 4 hr of incubation at 37°C. RBC GSH values were measured by the 5,5'-dithiobis-2-nitrobenzoic acid method of Beutler et al.\textsuperscript{14} In some experiments, the GSH stability of methemoglobin-containing RBC was studied. Methemoglobin was produced by incubation of 1 vol packed RBC with 1 vol isotonic sodium nitrite for 30 min at room temperature. The packed cells were
then washed three times with 8 vol isotonic saline and resuspended in glycerylglucoside buffer. Controls for these experiments were RBC incubated in saline at room temperature for 30 min and washed in a manner similar to that of the nitrite-treated cells.

\[^{14}C\text{-formate oxidation.}\] Since \[^{14}C\text{-formate}\] is oxidized to \[^{14}CO_2\] in the presence of \(H_2O_2\) and catalase, this system was also used to detect \(H_2O_2\).\(^{15}\) RBC were incubated in metabolic flasks as previously described, except that \[^{14}C\text{-formate}\] was used rather than \[^{14}C\text{-glucose}\]. Since there is good evidence that \(H_2O_2\) is preferentially reduced by glutathione peroxidase in RBC, \[^{14}C\text{-formate}\] oxidation was also studied in cells treated with \(N\)-ethylmaleimide (NEM) in amounts required to bind all intracellular GSH (2-3 \(\mu\text{mol NEM/ml RBC})^{16}\). NEM in these small amounts selectively blocks GSH.\(^{11}\)

\textit{Catalase inhibition technique.} RBC were incubated for 30 min with NEM as above. The cells were then washed with isotonic saline and incubated for an additional 2-4 hr with or without Adriamycin in the presence of 50 mM aminotriazole. In the presence of aminotriazole, hydrogen peroxide and catalase form an irreversible complex. Hydrogen peroxide generation can be detected by serial determination of catalase. Catalase was determined by the perborate-permanganate method described previously.\(^{12}\)

**RESULTS**

\textit{Effect of Adriamycin on the HMPS of RBC.} The rate of \(^{14}CO_2\) production from glucose-1-\(^{14}C\) by RBC suspensions incubated under various atmospheric conditions is shown in Fig. 1. After baseline shunt activity was established, Adriamycin was added in a concentration of 100 \(\mu\text{g/ml}\). In RBC suspensions incubated under air, addition of Adriamycin was followed immediately by a threefold increase in HMPS activity. This stimulation of the HMPS by Adriamycin did not occur when the experiment was performed under anaerobic conditions with a carbon monoxide atmosphere, indicating that this reaction requires oxygen. In order to determine if the stimulation of the HMPS by Adriamycin depended on molecular oxygen or, more specifically, on the presence of oxyhemoglobin, the experiment was also performed under an atmosphere of 6\(^\circ\)o carbon monoxide in air. A marked attenuation of the HMPS response occurred under these conditions, indicating a dependency on oxyhemoglobin for full activity. Similar results were obtained using daunomycin in the same concentration.

The effect of various concentrations of Adriamycin on peak HMPS activity of RBC under aerobic conditions is shown in Fig. 2. A definite dose-response
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Fig. 2. Effect of various doses of Adriamycin on peak HMPS activity of RBC suspensions. CO₂ production was calculated for steady-state conditions after addition of Adriamycin in concentrations shown. Each value, mean of three experiments (± 1 SD).

relationship was observed. In the remainder of the experiments a concentration of 100 µg/ml was used.

Addition of NEM in slight molar excess of measured RBC GSH levels reduced the HMPS stimulation produced by Adriamycin by 20% (Fig. 3). This indicates that a portion of the HMPS stimulation produced by Adriamycin is accounted for by oxidation of GSH. Another large portion of this stimulation occurs independently of the GSH pathway. Adriamycin and daunomycin thus produce stimulation of the HMPS of RBC by a mechanism that requires oxyhemoglobin for full activity and in part involves reduced glutathione.

Evidence for peroxide generation. GSH stability of RBC incubated with
Adriamycin or daunomycin with or without glucose is shown in Table 1. While RBC incubated with Adriamycin or daunomycin in the presence of glucose maintained normal levels of GSH, a significant fall in these levels occurred during incubation in the absence of glucose. No fall of GSH occurred under anaerobic conditions; likewise, no fall of GSH occurred when methemoglobin-containing RBC were used in the presence or absence of glucose. These latter observations again confirm the importance of oxyhemoglobin to this reaction.

Since 14C-formate is oxidized to 14CO2 in the presence of hydrogen peroxide and catalase,15 we used this system to search specifically for production of hydrogen peroxide in RBC in the presence of Adriamycin. No increase in formate oxidation occurred after addition of Adriamycin alone. Since hydrogen peroxide is reduced preferentially by glutathione peroxidase, however, rather than by catalase in RBC,10 we also studied formate oxidation in cells treated with NEM to bind GSH and thus allow the reaction of peroxide with catalase and formate to occur. Preincubation of RBC with NEM followed by addition
of Adriamycin resulted in marked augmentation of formate oxidation (from 0.004 to 0.034 ± 0.011 μmol ¹⁴CO₂/ml RBC/hr) (Fig. 4). Daunomycin produced similar results.

This generation of hydrogen peroxide by Adriamycin was confirmed also by the catalase-aminotriazole trapping technique. A marked fall of catalase (to less than 5% of control in each of three experiments) occurred in NEM-treated cells incubated with Adriamycin and aminotriazole for 4 hr.

DISCUSSION

These studies indicate that in human RBC Adriamycin and daunomycin cause oxidation of NADPH and thereby stimulate the HMPS. A portion of this reaction involves the oxidation of reduced glutathione. Another large portion of the shunt stimulation is not dependent on GSH but is dependent on the presence of oxyhemoglobin.

These observations suggest the following mechanism of action of Adriamycin and daunomycin in intact RBC: By undergoing cyclic oxidation-reduction, these agents may act as mediators of electron transfer from NADPH to oxyhemoglobin (Fig. 5). Oxidation of oxyhemoglobin with generation of superoxide occurs spontaneously to a minor extent and is enhanced by certain drugs. Superoxide presumably generated by this reaction may then undergo spontaneous dismutation to hydrogen peroxide. The direct oxidation of NADPH by Adriamycin and daunomycin would account for the larger portion of the shunt stimulation we observed. The hydrogen peroxide generated may react with GSH and provide further shunt stimulation. Bachur et al. recently presented evidence that in subcellular microsomal preparations a similar mechanism of electron transfer occurs. In that system, Adriamycin and daunomycin undergo cyclic oxidation-reduction through a free radical semiquinone intermediate with the transfer of electrons from NADPH to molecular oxygen.

We thus have shown that anthracycline-dependent electron transfer with generation of reactive oxygen compounds, previously demonstrated in subcellular organelles, also occurs in intact human erythrocytes. These findings have the additional clinical implication that if the ability of RBC to regenerate GSH is impaired, activated oxygen species generated by Adriamycin or daunomycin might cause cell damage by oxidation of protein sulfhydryls and peroxidation of membrane lipids. Thus individuals with glucose-6-phosphate dehydrogenase deficiency might be susceptible to hemolysis upon administration of anthracyclines, although such episodes have not been reported. Additionally, use of certain chemotherapy agents concurrently with anthracyclines might en-
hance oxidant injury; for example, bischloroethyl nitrosourea causes generalized deficiency of glutathione reductase. Although the concentrations of anthracyclines used here would not be achievable in normal human usage except perhaps transiently after intravenous administration, other drugs studied as models of oxidant hemolysis are used in much higher concentrations; in particular, phenylhydrazine has recently been shown to undergo similar reactions in erythrocytes.

The mechanism of enhanced oxygen radical generation suggested by our studies may apply to the cardiac myocyte, where the analogous oxyhemoglobin exists in large concentrations. The susceptibility of the heart to toxicity from anthracyclines might in part reflect less ability of myocardium than RBC to generate GSH and other antioxidants to defend against oxidant injury. The inability of cardiac myocytes, in contrast with erythroid marrow cells, to divide and replace damaged cells might account for the pattern of cumulative anthracycline-induced cardiac injury observed clinically. If the generation of activated oxygen species in heart tissue is indeed important in the production of clinical cardiac toxicity by anthracyclines, it remains unclear why this effect is peculiar to this one class of drugs, as opposed to other oxidant drugs in clinical use. In addition to obvious differences in dosage and in method of administration, there might well be differences in tissue uptake, intracellular transport and distribution, and reaction constants among the various oxidant drugs. On the other hand, certain other cytotoxic agents that generate reactive oxygen compounds also produce localized organ toxicity. These include neurotoxicity by 6-hydroxydopamine and pancreatic islet cell damage by alloxan, and pulmonary toxicity by paraquat. The reasons for the localization of the cytotoxicity produced by these oxidant drugs is likewise unclear.

Finally, it is likely that at least some of the therapeutic effects of anthracycline compounds may occur by free radical mechanisms. Recent evidence suggests that degradation of DNA by anthracyclines is oxygen dependent and may be inhibited by scavengers of oxygen compounds. Further study is needed of the possible role of oxygen radicals in producing the cardiotoxic and perhaps even the therapeutic effects of anthracyclines. The erythrocyte model we have described might be useful in the detection and screening of additional compounds capable of producing such radicals.

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