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ADRIAMYCIN CARDIOMYOPATHY: IMPLICATIONS OF CELLULAR CHANGES IN A CANINE MODEL WITH MILD IMPAIRMENT OF LEFT VENTRICULAR FUNCTION*

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Abstract—The present study has examined early cellular effects of chronic adriamycin administration to dogs using a protocol (1 mg/kg/week to a total cumulative dose of 240 mg/m^2) producing significant but small reductions in ejection fraction and stroke volume as determined echocardiographically prior to the development of clinical or radiological manifestations of heart failure. At this early phase of cardiomyopathy, significant reduction ($P < 0.05$) in sarcoplasmic reticulum Ca^{2+}, K^+-ATPase was observed without any change in mitochondrial, lysosomal or sarcolemmal marker enzymes. Myocardial calcium ($P < 0.01$) and glutathione ($P < 0.001$) levels were increased significantly. Detailed analysis of myocardial phospholipid profiles failed to show any significant differences between control and treated dogs. In contrast, red cell membranes showed increased phosphatidylcholine (PC) and decreased phosphatidylserine (PS) contents, resulting in a significant increase in PC/PS ratio ($P < 0.05$). No significant changes were detected in activities of catalase, superoxide dismutase or glutathione peroxidase in erythrocytes or myocardial tissue from control and adriamycin-treated animals. A significant ($P < 0.05$) elevation in plasma sialic acid was observed following adriamycin treatment. Our results suggest that early adriamycin-induced damage is unlikely to result from alterations in cellular processes protecting tissues against oxidant injury. Regression analysis indicated that, of the various abnormalities observed, only the elevated myocardial calcium levels and the increases in plasma sialic acid correlated with the degree of myocardial functional impairment. Our findings suggest the presence of sarcolemmal alterations in Ca^{2+} handling in early adriamycin-induced myocardial injury and indicate that measurement of plasma sialic acid should be further investigated as a possible noninvasive indicator of impending adriamycin cardiotoxicity.

The clinical use of anthracyclines in the treatment of various neoplastic diseases is limited by the risk of cardiotoxicity, which is related to the cumulative dose of drug administered and which is not always reliably predictable from non-invasive tests of myocardial function [1, 2]. A major factor determining the cardiotoxicity of the anthracyclines is thought to involve their conversion to reactive semiquinone intermediates which subsequently give rise to chemically reactive species, such as superoxide and hydroxyl radicals, capable of causing oxidative damage to membranes and alterations in subcellular organelle structural and functional integrity [3, 4]. Tissue defense mechanisms, which may be enzymatic or non-enzymatic in nature, serve to detoxify reactive oxidizing species or their potentially deleterious cellular oxidation products. Enzymes such as catalase, superoxide dismutase and glutathione peroxidase [5] and the radical scavenger glutathione [6] are present in a wide variety of tissues (including the myocardium and red blood cells) and likely play an important role in this regard. Increases, possibly adaptive, in the activities of catalase [7], superoxide dismutase and glutathione peroxidase [8] have been described in some tissues subjected to conditions of oxidative stress. However, reactive oxygen radicals may also impair the activities of these enzymes [9–11] and cause depletion of tissue glutathione [12]. It has been suggested that the vulnerability of the myocardium to anthracycline toxicity may arise from an intrinsic deficiency of these protective mechanisms in this tissue or possibly from an impairment in their functioning following treatment with these drugs [5, 13, 14].

Experimental studies of cardiotoxicity induced by adriamycin, the most commonly used of the anthracyclines, have provided evidence for drug-related alterations in the structural and functional integrity of several cellular components, including sarcoplasmic reticulum [15], mitochondria [16–18] and the plasma membrane [19–21]. Lipid peroxidation [3, 22], increased myocardial calcium levels [23], depletion of glutathione [24] and myocardial contractile abnormalities [25] are also demonstrable under certain experimental conditions. Efforts to relate such diverse findings to fundamental mechanisms governing clinical anthracycline-induced cardiotoxicity are limited by a number of considerations. Besides uncertainties relating to species differences [26, 27], the high doses of adriamycin employed in most experimental studies may obscure

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primary drug-related changes with secondary manifestations of tissue damage. Another major problem relates to difficulties in assessing the relevance of observations made *in vitro* using isolated subcellular fractions to the situation *in vivo*, given that adriamycin may be actively cytotoxic without entering cells [28]. These uncertainties are well illustrated by adriamycin-induced lipid peroxidation which is clearly demonstrable *in vitro* [29, 30], but whose role in mediating *in vivo* toxicity has been questioned recently [30–32].

With the foregoing considerations and limitations in mind, we have undertaken to investigate molecular aspects of chronic adriamycin cardiotoxicity *in vivo* using a drug administration protocol in dogs that produces a small, but significant, reduction in myocardial contractile function assessed by echocardiography. This experimental model should allow one to focus on early cellular alterations produced by minimally cardiotoxic doses of adriamycin both in myocardial tissue and in erythrocytes, which are known to accumulate anthracyclines to concentrations twice those in plasma [33]. The information so derived might not only provide further insights into the mechanism of adriamycin-induced cardiomyopathy, but also indicate the possible predictive value of red cell measurements in assessing the risk of its development clinically following repeated adriamycin administration.

METHODS

Experimental model. Adult mongrel dogs were treated for 10 weeks with adriamycin (1 mg/kg/week as a single intravenous injection) to a cumulative dose of 240 mg/m^2. Left ventricular function was assessed noninvasively in conscious unrestrained animals by two-dimensional echocardiography (Diasonics CV100 cardiac scanner using 5.0 MHz microsector probe) performed repeatedly throughout the course of drug treatment with the last measurements being taken 5 days prior to killing the animals. Four days prior to sacrifice, animals were anesthetized with pentothal (25 mg/kg) and chest X-rays were performed to determine whether or not there was any evidence of pulmonary congestion secondary to left ventricular failure. Ten days after the last injection, animals were again anesthetized and killed. This treatment produced the histological and ultrastructural changes characteristic of early adriamycin-induced cardiomyopathy described previously [34].

Analytical methods. Left ventricular samples taken from hearts of untreated and adriamycin-treated dogs were subjected to a variety of chemical and biochemical analyses, all of which have been described in detail in previous publications from our laboratory [35, 36]. Small tissue samples were used for the measurement of water content (drying to constant weight), inorganic cation composition (by atomic absorption spectrophotometry) and phospholipid profiles (two-dimensional thin-layer chromatography). Larger tissue samples were subjected to Polytron homogenization, differential centrifugation and sucrose density fractionation for the characterization of mitochondrial, sarcoplasmic reticulum, lysosomal and sarcolemmal alterations using the activities of specific marker enzymes as indices of subcellular organelle integrity [35, 36]. Other analyses on intact myocardial tissue included measurement of glutathione levels (using 5,5′-dithiobis-2-nitrobenzoic acid), and activities of catalase [37], superoxide dismutase [38] and glutathione peroxidase [39]. These latter measurements were also performed, for comparison, on liver tissue samples from these animals and, as well, on red cells obtained immediately prior to sacrifice.

Erythrocytes were studied for possible alterations in phospholipid profiles, membrane sulfhydryl group content, and in the activity of membrane-associated enzymes [40]. We have also compared the susceptibility of red cells from control and adriamycin-treated animals to oxidative stress *in vitro* by examining the effects of hydrogen peroxide on cellular levels of glutathione, malondialdehyde and methemoglobin as described in a previous report from our laboratory [41].

Statistical analysis. Each value represents mean ± S.D. in control (untreated) and adriamycin-treated dogs. Statistical analysis was performed by using Student's *t*-test. Linear regression analysis was used to assess correlation interrelationships among the various criteria of myocardial injury using a Compucorp statistical calculator.

RESULTS

Echocardiographic assessment of left ventricular function. Myocardial function was assessed echocardiographically prior to and during drug treat-

Table 1. Echocardiographic assessment of myocardial function in six dogs chronically treated with adriamycin

	Ejection fraction (%)	Stroke volume (ml)
Baseline	68 ± 6 (67 ± 6)	51 ± 13 (55 ± 10)
Adriamycin (240 mg/m^2)	51 ± 10* (49 ± 9†)	28 ± 7* (26 ± 6*)

Results are expressed as mean ± S.D. Value in parentheses in this and other tables represent recalculated mean ± S.D for five of the six treated dogs with the omission of data from one atypical animal near death at the time of sacrifice (see regression analysis in Table 9).

* $P < 0.01$, significant difference from baseline data prior to therapy.

† $P < 0.02$, significant difference from baseline data prior to therapy.

Table 2. Activities of subcellular market enzymes in control and adriamycin-treated dogs

Organelle	Marker enzyme	Activity	
		Control	Treated
Mitochondria	Azide-sensitive ATPase*	77.1 ± 22.7	79.0 ± 22.6
Mitochondria	Cytochrome oxidase†	−1.57 ± 0.26	−1.46 ± 0.25
Sarcolemma	Na^+,K^+-ATPase*	4.11 ± 0.94	4.27 ± 0.57
Sarcoplasmic reticulum	Ca^{2+},K^+-ATPase* (azide-insensitive)	9.53 ± 4.09	5.08 ± 0.83‡
		—	(5.10 ± 0.98‡)
		% Sedimentable activity	
		Control	Treated
Lysosomes	Acid phophatase	43.4 ± 7.1	52.4 ± 12.4
Lysosomes	Glucosaminidase	66.2 ± 2.4	70.9 ± 3.1

* Expressed in μmoles/hr/mg protein.
† ΔAbs at 550 nm/min/mg protein.
‡ Significant difference from control, $P < 0.05$.

ment. Measurements taken 5 days before killing the animals (Table 1) indicated that adriamycin administration produced small, but significant, reductions in ejection fraction in all animals, and these were associated with comparable reductions in stroke volume. These changes were not associated with any clinical or radiological evidence of congestive heart failure. This model might, therefore, be relevant to the earliest stages of clinical adriamycin-induced cardiomyopathy and, as such, was felt to represent a useful system with which to search for early predictive markers of this important limiting anthracycline toxicity. It should be mentioned that while five of the six adriamycin-treated dogs appeared to be in good physical condition immediately prior to sacrifice, one dog was markedly dehydrated, leukopenic and debilitated. In the tabular presentation of chemical and biochemical abnormalities resulting from adriamycin treatment, data from all the treated animals were used but in the case of statistically significant changes, values obtained by omitting the data from this one animal are included in brackets for comparison.

Biochemical functional integrity of subcellular organelles. A significant reduction in sarcoplasmic reticulum Ca^{2+}, K^+-ATPase was observed in adriamycin-treated animals (Table 2). However, there was no significant difference observed between control and adriamycin-treated dogs with respect to mitochondrial azide-sensitive ATPase and cytochrome oxidase activities. Similarly, sarcolemmal Na^+,K^+-ATPase was comparable in both groups. Lysosomal hydrolase sedimentable activity was unchanged by administration of adriamycin at these dose levels (Table 2).

Chemical analyses of intact myocardial tissue. Another measure of sarcolemmal integrity is obtained from analysis of myocardial tissue composition (Table 3). A significant increase in tissue calcium was observed in adriamycin-treated dog hearts. There were, however, no observed differences in water, Na, K or Mg contents between control and adriamycin-treated hearts. A significant increase in myocardial levels of glutathione was also observed following adriamycin treatment. No similar increase in glutathione content was present in liver tissue (results not shown).

Phospholipid analyses. Detailed analysis of myocardial lipid extracts from control and adriamycin-treated dogs failed to show any significant difference in levels of individual phospholipids (Table 4). In contrast, red cell membranes of adriamycin-treated

Table 3. Chemical analyses of intact myocardial tissue samples from control and adriamycin-treated dogs

	Water content (%)	Cation content (ng atoms/mg dry wt)				Glutathione content (μmoles/mg tissue)
		Na	K	Mg	Ca	
Control	78.3 ± 0.6	148 ± 35	247 ± 55	25.3 ± 2.5	4.4 ± 0.99	2.10 ± 0.24
Treated	79.1 ± 0.9	167 ± 14	245 ± 46	26.8 ± 3.2	8.45 ± 2.72*	2.89 ± 0.29‡
					(7.70 ± 2.50†)	(2.80 ± 0.19‡)

Results are expressed as mean ± S.D. Values in parentheses are mean ± S.D. for five of the six treated dogs (see legend of Table 1).
* $P < 0.01$.
† $P < 0.05$.
‡ $P < 0.001$.

Table 4. Phospholipid analyses of myocardial tissue, red cell membranes and plasma from control and adriamycin-treated dogs

	% Total lipid phosphorus						
	PC	Sph	PE	PS	PI	Cl	Ratio PC/PS
Myocardium							
Control	45.1 ± 3.2	3.1 ± 0.9	32.1 ± 3.0	2.6 ± 0.6	4.7 ± 0.4	11.1 ± 2.4	
Treated	44.4 ± 3.0	3.2 ± 1.0	32.9 ± 2.3	2.5 ± 1.1	4.7 ± 0.8	9.8 ± 1.8	
Red cell membranes							
Control	41.9 ± 2.2	13.4 ± 1.8	25.4 ± 1.2	16.7 ± 1.1	2.0 ± 0.7		2.53 ± 0.27
Treated	44.8 ± 2.3	12.9 ± 1.7	24.0 ± 1.1	14.9 ± 2.0	1.6 ± 1.3		3.16 ± 0.86* (3.39 ± 0.78*)
Plasma							
Control	84.0 ± 2.5	8.3 ± 2.1	1.3 ± 0.5	2.2 ± 0.3			
Treated	87.0 ± 2.4	7.6 ± 2.1	1.2 ± 0.6	1.3 ± 0.7			

Results are expressed as mean ± S.D.

Abbreviations: PC, phosphatidylcholine; Sph, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; and Cl cardiolipin.

* Significantly different from control, $P < 0.05$; values in parentheses are mean ± S.D. for five of the six treated dogs (see legend of Table 1).

dogs showed an elevation of phosphatidylcholine content and a decrease in phosphatidylserine, resulting in a significant increase in the phosphatidylcholine/phosphatidylserine ratio. An increase in the phosphatidylcholine content of plasma was noted in the drug-treated animals, but this did not quite attain statistical significance (Table 4).

Free radical-related processes. Increases in oxidative stress are associated frequently with elevations in the activities of one or more of the enzymes involved in the detoxification of the highly reactive species involved [7, 8]. The activities of catalase, superoxide dismutase and glutathione peroxidase from control and adriamycin-treated animals were measured in heart and, for comparison, in liver homogenates (Table 5). No significant changes were detected. Similarly, activities of red cell catalase, superoxide dismutase and glutathione peroxidase in the treated animals were indistinguishable from controls.

In another series of experiments, we have subjected red cells from control and adriamycin-treated animals to conditions of oxidative stress *in vitro*. The extent of conversion of hemoglobin to methemoglobin in red cell hemolyzates exposed to hydrogen peroxide was assessed from the absorbance increase at 630 nm [41] and was found to be unaffected by adriamycin treatment (results not shown). The concentration-dependent depletion of glutathione and increase in malondialdehyde levels in red cells exposed to hydrogen peroxide was also investigated in blood samples from control and adriamycin-treated animals. Again, no significant differences were detected (Table 6). Finally, no evidence for oxidative destruction of red cell membrane sulfhydryl groups could be found following adriamycin treatment (Table 7).

Red cell and plasma chemical analyses. A significant elevation in the sialic acid content of plasma was observed following adriamycin treatment (Table 7). Analyses of red cell and plasma cholesterol and

Table 5. Activities of catalase, superoxide dismutase and glutathione (GSH) peroxidase in control and adriamycin-treated dogs

	Catalase*		Superoxide dismutase†		GSH peroxidase ‡	
	Control	Treated	Control	Treated	Control	Treated
Myocardium	0.162 ± 0.063	0.218 ± 0.113	0.77 ± 0.09	0.80 ± 0.20	0.126 ± 0.012	0.115 ± 0.04
Liver	39.2 ± 3.8	39.2 ± 11.5	2.99 ± 0.57	3.33 ± 0.65	0.078 ± 0.014	0.066 ± 0.013
Erythrocytes	9.04 ± 5.17	12.8 ± 7.5	1.55 ± 0.25	1.59 ± 0.31	0.120 ± 0.024	0.105 ± 0.007

Results are expressed as mean ± S.D. of experiments with six dogs in each group.

* Units are "k" value × 10^{-4} per g tissue or per mg hemoglobin (for erythrocytes) as described in Ref. 37.

† Values represent units per mg tissue wet weight or per mg hemoglobin (for erythrocytes). One unit is that amount of enzyme activity causing 50% inhibition of the superoxide-dependent photolytic reduction of nitrobluetetrazolium (see Ref. 38).

‡ Activities are expressed as ΔAbs 340/min/mg for tissue homogenates or ΔAbs 340 nm/min/mg hemoglobin for erythrocytes (see Ref. 39).

Table 6. Susceptibility of red cells from adriamycin-treated and control animals to peroxide-induced damage *in vitro*

Glutathione depletion	Glutathione level (Abs at 412 nm/g red cells)	
$[H_2O_2]$ (mM)	Control	Treated
0	1.25 ± 0.21	1.11 ± 0.23
0.05	0.90 ± 0.18	0.75 ± 0.24
0.10	0.47 ± 0.14	0.47 ± 0.18
0.30	0.12 ± 0.11	0.12 ± 0.05
0.50	0.09 ± 0.05	0.10 ± 0.02
1.00	0.09 ± 0.04	0.10 ± 0.02
Malondialdehyde formation	Malondialdehyde level [ΔAbs (532–600) nm/g red cells]	
$[H_2O_2]$ (mM)	Control	Treated
0	0.04 ± 0.05	0.04 ± 0.06
10	5.08 ± 1.44	4.24 ± 1.31
20	7.44 ± 1.93	6.06 ± 2.28
30	8.12 ± 2.40	7.19 ± 2.11
40	8.28 ± 3.83	7.12 ± 2.37

All values are given as mean ± S.D.

phospholipid contents failed to reveal any differences between control and adriamycin-treated animals (Table 7).

Red cell membrane functional integrity. Activities of basal (Mg^{2+}-dependent) and Ca^{2+}-stimulated (Mg^{2+}-dependent) ATPases (representative of integral membrane proteins) were measured in membranes from control and adriamycin-treated animals. As well, kinetic properties of red cell actylcholinesterase (a peripheral membrane protein) were studied. No statistically significant differences between control and treated animals were found (Table 8).

Correlation of myocardial functional impairment with chemical and biochemical indices of injury. Potential relationships between adriamycin-induced reductions in myocardial contractility (as reflected in ejection fraction measurements) and other abnormalities found to be associated with chronic adriamycin administration were investigated. Of the various significant alterations observed here, only the elevations in plasma sialic acid and in myocardial calcium levels tended to correlate with the depressions in ejection fraction (Table 9). Correlation coefficients for variations in plasma sialic acid and for myocardial calcium level with respect to ejection fraction showed highly significant correlations (r values of -0.90 and -0.88 respectively) when data were recalculated, eliminating values obtained from a single adriamycin-treated dog who was severely debilitated and dehydrated, presumably as the result of an infection, at the time of sacrifice. The correlation coefficients for other abnormalities, such as the increased cardiac glutathione levels, the depression in sarcoplasmic reticulum ATPase activity and the increased PC/PS ratio in red cells did not show any appreciable improvement in r values with respect to ejection fraction under these conditions. As apparent from the tabular presentation of the data, omission of

Table 7. Chemical analyses of red cell membranes and plasma from control and adriamycin-treated dogs

	Phospholipid		Cholesterol		Sialic acid		Sulfhydryl titer	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Red cell membranes*	0.692 ± 0.066	0.700 ± 0.015	0.638 ± 0.065	0.639 ± 0.051	0.155 ± 0.024	0.147 ± 0.23	0.060 ± 0.005	0.057 ± 0.003
Plasma†	5.50 ± 0.7	5.57 ± 1.01	5.89 ± 1.07	5.07 ± 0.71	2.06 ± 0.22	3.17 ± 0.86‡ (3.04 ± 0.96‡)		

Results are expressed as mean ± S.D. Values in parentheses are mean ± S.D. for five of the six treated dogs (see legend of Table 1).
* In μmoles/mg protein.
† In μmoles/ml plasma.
‡ Significantly different from control at $P < 0.05$ level.

Table 8. Activities of erythrocyte membrane enzymes in control and adriamycin-treated dogs

	Mg^{2+}-ATPase	Ca^{2+}-ATPase	Acetylcholinesterase	
	(μmoles/hr/mg protein)		V_{max} (μmoles/hr/mg protein)	K_m (mM)
Control	0.406 ± 0.019	0.412 ± 0.046	0.162 ± 0.047	0.076 ± 0.006
Treated	0.434 ± 0.107	0.357 ± 0.049	0.152 ± 0.058	0.078 ± 0.005

Results are expressed as mean ± S.D. of experiments with six dogs in each group.

values obtained from the one atypical adriamycin-treated dog did not appreciably alter the mean values for the various abnormal chemical and biochemical properties noted in the treated group, and all differences remained significantly different from values for the control group.

DISCUSSION

Most earlier studies of experimental anthracycline-induced cardiotoxicity have investigated the effects of high cumulative doses of adriamycin which result in severe myocardial injury [42, 43]. Our aim in the present study was to search for early markers of cellular injury in both myocardial tissue and the blood to obtain a greater insight into primary mechanisms governing the cardiotoxicity, and also to suggest conveniently measurable predictive indices of incipient damage. Such chemical or biochemical markers, if present in red cells or plasma, might substantially improve the discriminating power of noninvasive testing to identify individuals at risk of developing anthracycline cardiotoxicity [44]. In the present study, dogs were given a low cumulative dose of adriamycin (240 mg/m^2) to produce a small, but significant, decrease in myocardial contractile function. Using this model of mild myocardial injury, two characteristic features of more severe anthracycline-induced cardiomyopathy are present, namely, the accumulation of intracellular calcium [23] and alterations at the level of the sarcoplasmic reticulum [15]. Other chemical and biochemical changes which might have been expected on the basis of previous *in vivo* and *in vitro* studies of adriamycin cardiotoxicity were not observed. Most notable among these was the absence of detectable lipid peroxidation and of alterations in mitochondrial cytochrome oxidase activity. Our studies suggest that early adriamycin damage is unlikely to result from a generalized impairment in cellular processes serving to protect tissues against oxidant injury.

Acute administration of high doses of adriamycin to rats (15 mg/kg) or rabbits (7 mg/kg) but not mice (15 mg/kg) has been shown previously not to alter myocardial levels of catalase or glutathione peroxidase [5, 31, 45]. However, tissues can respond adaptively with increases in the activity of catalase, superoxide dismutase and glutathione peroxidase under cetain conditions [7, 8]. We found no changes in the activity of these three enzymes either in the myocardium or in red cells of dogs treated chronically with adriamycin. Rats treated chronically with adriamycin for several weeks showed no changes (relative to controls) in myocardial superoxide dismutase activity, levels of lipid peroxidation products or glutathione content [46].

Glutathione levels in red blood cells and myocardium have been shown to be transiently reduced in mice following acute adriamycin administration (15 mg/kg) with return to control values in 24 hr [24]. We found no change in red cell or liver glutathione in adriamycin-treated dogs, but myocardial levels were elevated significantly. While this discrepancy may relate to differences in species (mouse versus dog) or in the acute versus chronic drug administration protocols, it may be that, in view of the relatively mild injury present in our experimental model, the increased level of glutathione could indicate an initial compensatory response which might be followed later by progressive glutathione depletion as the result of its consumption in the enzymatic and non-enzymatic detoxification of reactive radicals. The recent finding that livers from rats treated chronically with ethanol have elevated levels of hepatic glutathione in association with an increased lipid peroxide content may be another example of early adaption to conditions of increased oxidative stress [47].

Table 9. Regression analysis of the relationship between depression in ejection fraction and other abnormalities associated with adriamycin treatment

	Myocardium			Plasma	Erythrocyte membranes
	Glutathione content	Ca^{2+} content	SR-Ca^{2+} ATPase activity	Sialic acid level	PC/PS ratio
r (N = 6)	+0.145	−0.257	+0.014	−0.558	+0.258
r^* (N = 5)	−0.447	−0.880	+0.152	−0.900	+0.278

* These r values were determined using data obtained from five of six treated dogs with the omission of data from one atypical animal near death at the time of sacrifice.

On the basis of experiments showing that the exposure of red cells to adriamycin *in vitro* can lead to hemoglobin oxidation and the production of superoxide, hydrogen peroxide and reactive hydroxyl radicals [48, 49], we have hypothesized an increased susceptibility of red cells to oxidative stress which might be detected in membrane phospholipid peroxidation of red cells from animals receiving adriamycin chronically. We have found no evidence for oxidative damage in red cells from adriamycin-treated animals, emphasizing that the results of *in vitro* experiments with adriamycin are not necessarily predictive of its effects *in vivo*. This is well illustrated by the ability of adriamycin, especially in the presence of iron and/or a deficiency of vitamin E, to stimulate lipid peroxidation in a variety of *in vitro* systems [3, 22, 29, 30]. Although lipid peroxidation has previously been assigned a primary role in anthracycline cardiotoxicity *in vivo*, there is now considerable experimental evidence (in addition to that obtained here) which fails to support this hypothesis, including the failure of vitamin E and/or elenium administration to prevent cardiac lesions associated with chronic adriamycin administration [30–32, 45].

Cardiolipin is known to be an essential co-factor for the mitochondrial enzyme cytochrome oxidase [18, 40]. Recent studies *in vitro* have shown that adriamycin can mediate electron transport between NADH and cytochrome *c* resulting in the formation of a covalent complex between adriamycin and cardiolipin [51]. Experiments using pig heart submitochondrial particles have also demonstrated that adriamycin causes rapid inactivation of cytochrome oxidase as well as a loss of cardiolipin and phosphatidylethanolamine. This process requires Fe^{3+} and, since intracellular levels of this ion are normally very low, there was some question raised as to the occurrence of such changes *in vivo* [30]. This would be consistent with the results of the present study, in that adriamycin treatment had no detectable effect either on the activity of cytochrome oxidase in myocardial mitochondrial fractions or on the recovery of cardiolipin and phosphatidylethanolamine in myocardial tissue extracts. Our findings raise the possibility that alterations in mitochondrial morphology and function observed following adriamycin treatment *in vivo* may not be direct, but rather are indirect effects of the drug, possibly resulting from changes induced by the interaction of the drug with the plasma membrane [20, 21, 28].

Again, on the basis of *in vitro* experiments, where adriamycin has been shown to exert an inhibitory effect on cardiac microsomal Na^+, K^+-ATPase [21], comparable effects following adriamycin treatment *in vivo* might have been anticipated, but no decrease in sarcolemmal Na^+,K^+-ATPase was detected. However, the approximately 2-fold elevation in mean myocardial calcium level that we have observed following adriamycin treatment indicates the presence of a relatively specific alteration in sarcolemmal integrity early in the course of the cardiomyopathy. This is consistent with the results of earlier experiments by Olson *et al.* [23] in rabbits treated chronically with adriamycin where it was demonstrated that an approximately 1.5-fold increase in myocardial calcium was already present prior to the development of the cardiomyopathy while a greater elevation (2.7-fold) was found in animals with evidence of cardiomyopathy [23]. Although the mechanism underlying this sarcolemmal defect is unknown, the greater propensity for calcium changes distinguishes it from the more generalized change in ion permeability associated with ischaemia/reperfusion injury [36]. It has been suggested that adriamycin can increase calcium influx into ventricular cells by stimulation of voltage-dependent calcium channels [52]. However, adriamycin has also been shown to inhibit Na^+/Ca^{2+} exchange in dog heart sarcolemmal vesicles [53], and there has been some suggestion that adriamycin may also decrease passive membrane permeability to calcium [54]. Thus, adriamycin-induced elevations in myocardial calcium levels likely result from a complex interplay of factors which has yet to be clarified.

Adriamycin is known to interfere with a number of Ca^{2+}-dependent processes [25, 54–56], and its ability to cause a continuous dose-dependent prolongation of contraction in isolated rabbit heart papillary muscle has been suggested to arise from a drug-induced impairment in Ca^{2+} handling at the level of the sarcoplasmic reticulum [57]. Our finding of a depression in the activity of azide-insensitive, ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA)-inhibitable Ca^{2+},K^+-stimulated ATPase in the hearts of adriamycin-treated dogs is entirely consistent with this possibility. Further, the lack of detectable effect of drug treatment on the Ca^{2+}-stimulated ATPase of erythrocyte membranes would tend to argue against a direct effect of adriamycin on the sarcoplasmic reticulum enzyme. Rather, the observed decrease may result from the generation of reactive radical species as the result of enzymatic NADPH/cytochrome P-450-dependent reduction of the anthracycline at the level of the sarcoplasmic reticulum [3, 4]. In this regard, it has been shown recently that, in the presence of phorbol myristate acetate-stimulated human leucocytes, isolated canine myocardial sarcoplasmic reticulum preparations exhibit reductions in both Ca^{2+} transport and Ca^{2+}-ATPase activities. Experiments with specific scavengers have implicated hydroxyl radicals in the inactivation of the ATPase and hydrogen peroxide in the depression of Ca^{2+} uptake [58]. Although the mean activity of sarcoplasmic reticulum Ca^{2+}-ATPase was reduced significantly relative to control in the adriamycin-treated animals as a group, no correlation was apparent between the degree of reduction in ejection fraction and corresponding Ca^{2+}-ATPase activities. This may indicate that the impairment in sarcoplasmic reticulum Ca^{2+}-ATPase is not the sole or the major determinant of the decrease in myocardial contractility in the present model of early adriamycin cardiotoxicity. The ability of anthracyclines to decrease the fast-exchanging component of sarcolemmal-bound calcium has been implicated in the impairment of myocardial contractility early in the course of anthracycline-induced cardiomyopathy [59].

Finally, with regard to our search for conveniently measurable chemical or biochemical indices of adriamycin cardiotoxicity in blood, we found that adriamycin-treated animals exhibited elevations in plasma sialic acid which paralleled the degree of myo-

cardial damage. It is well known that sialyltransferase is increased in the circulation of cancer patients [60] and that plasma sialoglycoproteins may serve as non-specific tumour markers and may be useful as predictors of disseminated breast cancer [61] or malignant melanoma recurrence [60] and as an index of tumor burden [62]. Thus, while our observations suggest the potential value of plasma sialic acid measurements in assessing risk of adriamycin cardiomyopathy, further information on the complex interplay between effects of adriamycin and neoplastic processes on plasma sialoglycoprotein levels will be required before the clinical usefulness of such measurements can be evaluated.

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