ANTIOXIDANT PROTECTION AGAINST ADRIAMYCIN-INDUCED CARDIOMYOPATHY

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    European 0335
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    United States 0337
History of Science 0585
Law 0398
Political Science
    General 0615
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        Relations 0616
    Public Administration 0617
Recreation 0814
Social Work 0452
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    General 0626
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        Therapy 0354
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    Optics 0752
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    Solid State 0611
Statistics 0463
Applied Sciences
Applied Mechanics 0346
Computer Science 0984

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    General 0537
    Aerospace 0538
    Agricultural 0539
    Automotive 0540
    Biomedical 0541
    Chemical 0542
    Civil 0543
    Electronics and Electrical 0544
    Heat and Thermodynamics ... 0348
    Hydraulic 0545
    Industrial 0546
    Marine 0547
    Materials Science 0794
    Mechanical 0548
    Metallurgy 0743
    Mining 0551
    Nuclear 0552
    Packaging 0549
    Petroleum 0765
    Sanitary and Municipal 0554
    System Science 0790
Geotechnology 0428
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Clinical 0622
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BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. ABSTRACT

Adriamycin, an anthracycline antibiotic is very effective against many soft and solid human malignancies. However, the potential usefulness of this potent chemotherapeutic agent is limited by the occurrence of a dose dependent cardiomyopathy and congestive heart failure which is generally refractory to inotropic interventions. Although the mechanism of adriamycin-induced cardiomyopathy may be an interplay of different factors, a relative increase in free radical stress has been suggested to play a major role. In this study we examined the beneficial effects of probucol, a lipid lowering drug with strong antioxidant properties, on the development of adriamycin cardiomyopathy and congestive heart failure.

Adriamycin (ADR) was administered in rats in six equal injections (i.p.) over a period of two weeks (cumulative dose of 15 mg/kg). After three weeks of post-treatment, cardiomyopathy and congestive heart failure were characterized by ascites (about 90 ml in each animal), enlarged liver and mortality of 30%. Hemodynamic measurements revealed depressed cardiac function. Left ventricular peak systolic pressure (LVPSP) was decreased up to 30% and left ventricular end diastolic pressure (LVEDP) was increased up to six times compared to controls. Ultrastructural studies showed severe myocardial cell damage typical of adriamycin cardiomyopathy. It was characterised by vacuolization in cytoplasm, loss of myofibrils, swelling of mitochondria and dilation of the sarcotubular system. Myocardial antioxidant enzyme glutathione peroxidase (GSHPx) activity was decreased by 32%. Superoxide dismutase (SOD) and catalase (CAT) did not change.

Lipid peroxidation as indicated by malondialdehyde (MDA) levels was about 80% higher than in the control group.

Probucol (Cumulative dose 60 mg/kg) was administered i.p. in six equal injections over a 2 week period on days alternating with ADR treatment. In these concurrently treated probucol + ADR treated animals, significant attenuation of myocardial ADR effects was found. Average amount of ascites was reduced to 20 ml per animal and mortality rate in this group was reduced to 10%. Hemodynamic parameters were at control levels with the exception of LVEDP, which was about three times higher than normal. Morphological studies done on the hearts from probucol and ADR treated animals showed regular myofibrillar structure, maintained sarcotubular reticulum and preserved mitochondria. However, some perimitochondrial edema was noticeable. Probucol treatment alone increased SOD activity by 30% and in combination with ADR the activity was 90% higher than in the control animals. Myocardial GSHPx and Catalase activities in the PROB and PROB + ADR groups were comparable to control animals. Lipid peroxidation, measured by MDA levels was back to normal.

A pre treatment with probucol for two weeks (cumulative dose 60 mg/kg) in addition to the concurrent treatment, employing the same animal model as described above resulted in complete prevention of pathological, functional and structural changes due to adriamycin. There was no mortality or ascites in these animals. In PROB + ADR group, both GSHPx and SOD activities were significantly higher than control.

These data provide evidence that ADR cardiomyopathy is associated with an antioxidant deficit. Improved cardiac structure as well as function due to treatment with probucol may be related to the maintenance of the antioxidant status of the heart. Protocol involving pre as well as concurrent treatments with probucol was found to be more effective in providing myocardial protection against adriamycin. The study suggests potential usefulness of antioxidant (probucol) therapy in ADR cardiomyopathy.

II. INTRODUCTION

Adriamycin (also known as doxorubicin) is a potent antitumor antibiotic, used in the treatment of a variety of soft and solid human malignancies. The treatment is characterized by acute as well as chronic adverse side effects. Acute changes included myelosuppression, nausea, vomiting and arrhythmias. These acute side effects are reversible and/or clinically manageable and do not present a major problem. The chronic effects on the other hand, are characterized by myocardial cell damage and congestive heart failure. Furthermore, adriamycin-induced heart failure is refractory to inotropic drugs and mechanical circulatory assist devices. Thus, usefulness of adriamycin as an antitumor drug is limited by the risk of developing heart failure. It is therefore, important to understand the pathogenesis of adriamycin-induced cardiomyopathy and failure to reduce the risk of this side effect.

In a retrospective study of a patient records, a cumulative dose of 550 mg/m² body surface area of adriamycin was found to be associated with a significant number (30%) of patients developing congestive heart failure. Development of adriamycin-cardiomyopathy was found to be a dose-dependent phenomenon and thus a clinical dosage limit of 550 mg/m² has been suggested to minimize the risk of heart disease. Cardiomyopathic changes in the heart found after chronic treatment with adriamycin, include dilation of the heart, focal degeneration, atrophy of myocytes and fibrosis. Typical morphological changes of cardiac cells include cytoplasmic vacuolization due to distention of the sarcoplasmic reticulum, loss of myofibrils, disruption of sarcomeres, swelling and lysis of mitochondria, margination of chromatin along the

4

nuclear membrane and intracellular edema.

Research on adriamycin-induced myocardial dysfunction has resulted in several postulates including interaction of adriamycin with deoxyribonucleic acid and inhibition of nucleic acid as well as protein synthesis, release of vasoactive amines, changes in adrenergic mechanisms, abnormalities in high energy phosphate metabolism, free radical formation and lipid peroxidation, alterations in sarcolemma and membrane bound enzymes, increase in lysosomal number as well as enzymes, imbalance of myocardial electrolytes and occurrence of calcium overload. Because of the semiquinone moiety in the adriamycin molecule, it is reported to increase the oxygen radical activity which can cause peroxidation of polyunsaturated fatty acids thus compromising membrane function.

Present study was undertaken to test the hypothesis that a relative increase in oxidative stress during adriamycin-induced cardiomyopathy may be due to antioxidant deficit and that supplementation with an exogenous antioxidant may prevent or reduce effects of adriamycin. Probucol, a well known lipid lowering drug, was used as an exogenous antioxidant. The reason for choosing probucol was its strong antioxidant properties; it contains two phenolic groups in contrast to one phenolic group present in vitamin E, a naturally occurring antioxidant.

Cardiomyopathy and heart failure were induced in rats by a subchronic treatment with adriamycin. For examining the effects of probucol, two different treatment protocols were followed. In one approach, concurrent treatments with probucol and adriamycin were given over a period of two weeks and in the other, this

5

protocol was preceeded by two weeks of treatment with probucol alone. General condition of the animals, food consumption and body weight changes as well as mortality were monitored daily. Hemodynamic function was assessed in animals and hearts were examined for ultrastructure, lipid peroxidation and endogenous antioxidants. Present study shows occurrence of endogenous antioxidant deficit due to adriamycin and probucol has a beneficial effect with respect to the cardiac antioxidant reserve as well as structure and function.

III. LITERATURE REVIEW

A. GENERAL BACKGROUND

Daunomycin (daunorubicin), an antibiotic with powerful antitumor activity, was isolated from strains of <u>Streptomyces peucetius</u> in Italy, in the early 1960's (Arcamone et al., 1964). Soon after that, a related drug doxorubicin (adriamycin) was isolated from a mutant of <u>streptomyces peucetius</u> and was found to be more potent than daunorubicin. A number of experimental and clinical studies on different neoplasms showed good results with adriamycin in terms of reduction in the tumor size as well as number (Di Marco, 1969; Gilladoga, 1976; Lefrak et al., 1973).

In the early seventies, it became obvious that chronic use of adriamycin has some serious cardiotoxic side effects. In a retrospective study of 366 patient records, it was shown that congestive heart failure developed in 30% of the patients once the total cumulative dose of adriamycin exceeded 550 mg/m^2 body surface area (Lefrak et al., 1973). Dose dependency of the incidence of heart failure was also demonstrated by other subsequent studies (Praga et al., 1979). An empirical dose of 550 mg/m^2 of adriamycin was suggested as a threshold in clinical use (Lefrak et al., 1973).

B. ADRIAMYCIN AND PHARMACOKINETICS

Adriamycin is a tetracyclic aglycone to which an amino sugar is attached through a glycosidic bond. It is obtained either by aerobic fermentation of <u>Streptomyces peucetius</u> or by chemical synthesis from daunomycin (Arcamone, 1972). The drug is available in Canada as Adriamycin hydrochloride. Adriamycin is readily

7

soluble in water, physiological saline and methanol, but is only slightly soluble or insoluble in less polar solvents (Arcamone, 1972). In its lyophilized form adriamycin is stable at room temperature for years without any loss of activity. Even in solution (pH 7.0), it is stable for more than a month. Adriamycin is rapidly cleared from the plasma and accumulated in different tissues including heart, and excreted slowly (Kimura, 1972; Yesair, 1972). Half life in myocardial tissue is about 48 hours and 40-50% of the drug is metabolized in the liver and excreted in bile. Smaller amounts are excreted via the kidneys in urine (Benjamin, 1975; Bachur, 1974, 1979). Adriamycin is not absorbed by the gastrointestinal tract (Arena et al., 1972).

C. ADRIAMYCIN DISTRIBUTION AND METABOLISM

Adriamycin, because of histophillicity, penetrates rapidly into cells and nuclei (Buja, 1973; Egorin, 1974). The mechanism of adriamycin transport is complex and not completely understood, possibly because the drug can permeate the membrane in several different ways, including both carrier-mediated transport and simple diffusion (Skovsgaard et al., 1982). After entering the nuclei, adriamycin intercalates between the base pairs of DNA, and its antitumor activity is due to the inhibition of nucleic acid and protein synthesis (Arena et al., 1974).

During adriamycin metabolism in the liver, adriamycinol, the major metabolite of adriamycin is formed. Adriamycinol demonstrated a more toxic effect on the heart than did adriamycin in as much as it showed a higher inhibitory effect on cardiac contractility, calcium and Na^+/K^+ pump of sarcolemma and Mg-dependent ATP-ase in submitochondrial vesicles (Boucek et al., 1987). The same study

8

demonstrated that adriamycin could be converted to adriamycinol in cardiac tissue _in vitro_. It suggested that the accumulation of potent cardiotoxic adriamycinol in the myocardium could be largely responsible for the toxicity.

D. RISK FACTORS

While majority of patients should not receive more than 550 mg/m^2 of adriamycin in order to avoid its cardiotoxic side effects, there are patients who could tolerate higher doses of the drug with no apparent side effects. On the other side, presence of other risk factors can potentiate the cardiotoxic effects of the drug at a cumulative dose much below the suggested upper limit (Bristow et al., 1978). Examination and determination of different risk factors for development of adriamycin-induced cardiomyopathy has also been done (Minnow et al., 1977). Some of the proposed risk factors are as follow:

D.1. Radiation Therapy

Radiation therapy, previous and concomitant, is thought to potentiate adriamycin-induced cardiac damage. In this regard, latent radiation-induced cardiac changes were potentiated by adriamycin treatment (Billingham et al., 1977). Previous mediastinal radiation therapy has also been shown to lower the cumulative dose of adriamycin that can induce cardiomyopathy at a dose below the recommended dosage limit of 550 mg/m^2 body surface area (Fajardo et al., 1976; Minow et al., 1977; Billingham et al., 1979). A synergistic effect of adriamycin and mediastinal radiation was also reported (Fajardo et al., 1976). These authors observed that animals receiving both adriamycin and radiation treatment developed more extensive cardiac

9

lesions than animals receiving only one or the other of the treatments. These studies suggested that patients with previous or concomitant radiation therapy should receive adriamycin in the total dose well below 550 mg/m² and that possible development of cardiomyopathy in these patients should be monitored very closely.

D.2. Liver disease

The highest concentrations of adriamycin and its metabolites are detected in the lung, kidney, spleen and liver (Bachur, 1975). In the metabolism of adriamycin, biliary excretion plays a very important role. If the biliary excretion is compromised, adriamycin will accumulate in the body and cause greater drug toxicity. Study of a patient with severe liver disease receiving adriamycin revealed elevated plasma levels of adriamycin (Benjamin et al., 1974). These patients were prone to fatal and life-threatening conditions. Based on these findings, it was suggested to decrease the adriamycin dose in patients with liver disease (Benjamin, 1975).

D.3. Combination Chemotherapy

During the chemotherapy, adriamycin is very often used in combination with other drugs such as cyclophosphamide, vincristine and bleomycin. Adriamycin-cyclophosphamide treatment was found to potentiate adriamycin cardiotoxicity in the dosage of adriamycin below the limit of 550 mg/m² (Minow et al., 1977). However, opposite findings were reported by Bristow et al., (1978). They could not find that concurrent cyclophosphamide-adriamycin therapy would increase the risk of adriamycin-induced cardiomyopathy. In any case, for the patients receiving cyclophosphamide concurrently with adriamycin, it is advisable to reduce adriamycin

10

cumulative dose of 450 mg/m^2 body surface area. Bleomycin and vincristine were also found to be associated with higher incidence of the occurrence of a refractory adriamycin cardiomyopathy (Praga et al., 1979).

D.4. Previous Cardiovascular Disease

Existence of previous e.g. electrocardiogram abnormalities and hypertension was also found to be associated with increased incidence of adriamycin-induced cardiomyopathy (Praga et al., 1979). Another retrospective study of patients' records also demonstrated that the probability of developing adriamycin-induced cardiomyopathy was higher in patients with previous cardiac disease or hypertension (Von Hoff et al., 1979). In an animal model, cardiac hypertrophy subsequent to aortic banding resulted in cardiotoxicity and structural damage to the hypertrophied heart at a much lower cumulative dose (5 mg/kg body weight) of adriamycin (Singal, 1983; Singal et al., 1984) while no damage was observed in sham control animals given the same dose of the drug (Singal, 1983). These studies indicated an increased risk of adriamycin cardiomyopathy possibly due to an altered metabolic state (Singal, 1985).

D.5. Calcium Entry Blockers

A number of studies investigated the effects of myocardial calcium accumulation in the pathogenesis of adriamycin-induced cardiomyopathy. Effects of verapamil and nifedipine, the calcium channel blockers, have been studied in animals treated with adriamycin and the results have been controversial. In this regard, verapamil has been shown to be protective against adriamycin-induced

11

cardiomyopathy by one group (Daniels et al., 1976) and accentuation of cardiotoxic effects of adriamycin by verapamil has been shown by others (Young et al., 1976). The suggestion that calcium channel blockers verapamil and nifedipine do not prevent but accentuate adriamycin-induced cardiomyopathy was also reported (Klugman et al., 1981). This aspect has been a subject of a review report (Rabkin and Godin, 1985). It is possible that Ca^{2+} overload is prevented by verapamil at high concentrations but other cardiovascular effects of the antagonist may become the determining factor.

D.6. Age

By the end of the seventies, a large retrospective study of 4018 patient records was done trying to correlate several different factors with the probability of developing drug induced heart failure at various adriamycin dose levels (Von Hoff et al., 1979). Comparing different age groups, they observed a steady increase in the probability of developing congestive heart failure with increasing patient age. In another study, it was noted that patients of age over 70 and with increasing the dose of adriamycin appear to concomitantly increase the probability of developing congestive heart failure (Bristow et al., 1978). However, Praga et al., (1979), found no statistical significance in age as a risk factor in a study of 1273 patients. In an animal model, increase in mortality as well as myocardial cell damage were demonstrated with age (Weinberg and Singal, 1986; Deally and Singal, 1990).

E. CARDIOVASCULAR SIDE EFFECTS

Adriamycin induced cardiovascular changes have been observed in

experimental animals as well as in patients. They can be categorized into acute and chronic effects. The acute effects develop within minutes or hours after the intravenous administration of the drug. These effects are not life threatening and are reversible or clinically manageable. Chronic effects, on the other hand, develop after several weeks or months of treatment and even after the completion of therapy. The most serious chronic effect is the insidious onset of a dose-dependent cardiomyopathy often leading to congestive heart failure (Buja et al., 1973; Chalcroft et al., 1973; Lefrak et al., 1973; Jaenke et al., 1974). This chronic cardiotoxic side effect has limited the use of adriamycin.

E.1. Acute Cardiotoxicity

Acute cardiotoxic side effects of adriamycin include hypotension, tachycardia and different electrocardiographic abnormalities (Herman et al., 1971; Arena et al., 1972; Lefrak et al., 1973; Zbinden and Brandle, 1975). In rabbit and dog, tachycardia, inversion of the T-wave (Arena et al., 1972; Bachmann et al., 1975) and reduction in the amplitude of EKG waves were reported (Herman et al., 1971). Addition of a number of tubulin-binding agents (vinblastin, vincristine, navelbine) to rhythmic, spontaneously pulsating rat cardiac cells immediately reversed adriamycin-induced arrhythmias (Lampidis et al., 1992). Adriamycin administrations (1.5 mg/kg) at 10 minutes was shown to have a negative inotropic effect and caused a decrease in both aortic and coronary blood flow in the dog heart. A negative inotropic effect of adriamycin (5 mg/ml) was also demonstrated in isolated heart preparations. This effect was not reversed by popranolol or quinidine (Arena et al., 1972). Breed et al.,

(1979) suggested that the decline of the contractile properties in the hearts of both rats and rabbits treated with adriamycin was dose-related, and that the decline in contractility was progressive. In the in vitro studies, at low drug concentrations, adriamycin has been found to increase the contractility in guinea pig atria (Villani et al., 1978), chick heart (Azuma et al., 1981) and isolated papillary muscle (Van Boxtel et al., 1978). In rat papillary muscle exposed to a wide range of concentrations of adriamycin (1 nM to 1 mM) no stimulation was seen at lower doses but a clear depressant effect was observed at higher concentrations (Singal and Pierce, 1986). From these in vitro studies it appears that a positive inotropic effect or no effect is seen at low drug concentrations (Van Boxtel et al., 1978; Von Hoff et al., 1979; Kim et al., 1980), while a negative effect was observed at higher concentrations of the drug (Singal and Pierce, 1986).

Shortly after administration (as early as 10 minutes after a single injection of 10 mg/kg, adriamycin), adriamycin caused nuclear segregation in mouse myocardium (Lambertenghi-Deliliers, 1976). These nuclear changes have been attributed to the interaction of the drug with nuclear DNA. Reversibility of these changes is still controversial as nuclear changes were reported to be reversed within 14 hours (Lambertenghi-Deliliers, 1976). Furthermore, only a small percentage of myocardial cells in patients who died from chronic anthracycline toxicity showed nuclear lesions (Buja et al., 1973). However, in rats injected with a higher dose of adriamycin (40 mg/kg) the nuclear changes did not return to normal (Merski et al., 1976). These differences in nuclear changes may be due to age, species differences and/or the

14

amount of drug administered.

Hemodynamic changes due to acute effects of adriamycin were demonstrated in an open chest dog heart perfusion preparation (Bristow et al., 1980). There were dose related decreases in cardiac output, mean blood pressure, left ventricular end diastolic pressure (LVEDP) and peripheral vascular resistance, as well as increase in heart rate. These hemodynamic changes could be reproduced by infusion of histamine or prevented by a histaminergic blocker (Bristow et al., 1980). The same authors suggested that histamine, or histamine plus catecholamines or protaglandins could contribute to the pathogenesis of adriamycin cardiomyopathy. In chronic adriamycin treated rabbits mean blood pressure (Arnolda et al., 1986; Wanless et al., 1987) and cardiac output dropped significantly and total peripheral vascular resistance and heart rate increased dramatically (Arnolda et al., 1986; Griffin-Green et al., 1988). Slopes of Frank-Starling curves were flatter in adriamycin treated rabbits (Wanless et al., 1987). Circulating norephinephrine and renin levels were elevated in the chronic model (Arnolda et al., 1986). These results suggest that in the early stage of administration of adriamycin, hemodynamic changes may be due to the adriamycin stimulated histamine release, reduced peripheral resistance and a decrease in circulating blood volume. With the development of cardiomyopathy, physiological compensatory mechanisms are leading to increased peripheral vascular resistance and a redistribution of blood supply to major organs, resulting in the low output congestive heart failure.

E.2. Chronic Cardiotoxicity

Chronic administration of adriamycin produces typical cardiotoxicity. Extensive morphological destruction is always accompanied by congestive heart failure which does not respond to therapy and becomes progressively worse (Lefrak et al., 1973; Buja et al., 1973; Cortes et al., 1975; Von Hoff et al., 1979; Bristow et al., 1978). In a comprehensive retrospective study, occurrence of heart failure in patients treated with adriamycin was reported (Lefrak et al., 1973). It was found that this drug associated heart failure was dose-dependent. The incidence increased rapidly at doses above 550 mg/m^2 body surface area. A total of 399 patients treated for far-advanced carcinoma by repeated injections of adriamycin (total cumulative dose 505 to 1004 mg/m^2 body surface area) over a period of several months showed several characteristic changes: marked hypotension (B.P. 70/50 mm Hg), tachycardia (150 beats /min) and a conspicuous decrease in the QRS voltage on the EKG, cardiac dilation and ventricular failure.

The heart failure was marked by total refractoriness to inotropic drugs and mechanical circulatory assistance. In experimental animals, repeated injections of adriamycin caused a depression in cardiac function and the response of these animals to norepinephrine (2 and 10 mg/kg) or epinephrine (0.5 and 1 mg/kg) was blunted (Zbinden et al., 1978; Tong et al., 1991). After chronic exposure to adriamycin, the contractile responses of single isolated rabbit cardiac myocytes to calcium and isoprenaline were depressed, but relaxation velocity in cells from adriamycin treated animals remained unaltered (Jones et al., 1990). Similar findings were reported by

16

Gorodetskaya et al., in 1990. They reported no inotropic response to isoprenaline administration in rats treated with adriamycin (2 mg/kg once a week for 10 weeks). Reduced responsiveness to epinephrine (x 10^{-5} M) and Ca^{2+} was also reported in 1 1/2 and 6 months old rats treated with adriamycin (Weinberg and Singal, 1986). In an in vivo dose-response study, epinephrine-induced cardiovascular response was significantly attenuated (Tong et al., 1991). In vitro studies on the effects of adriamycin have confirmed depressed contractile function (Azuma et al., 1981; Singal and Pierce, 1986).

The increase in serum glutamic oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH) and creatine phosphokinase (CPK) have been reported to accompany heart failure (Lefrak et al., 1973). The release of these enzymes which are present in high concentrations in myocardial cells indicated the changes in cell membrane permeability. The elevations in serum enzyme levels provide evidence concerning myocardial cell membrane abnormality and changed membrane permeability in cardiomyopathy due to adriamycin treatment. In vitro studies have lent support to these findings (Singal and Panagia, 1984). Altered intracytoplasmic concentrations of ions found in adriamycin cardiomyopathy are also suggesting altered membrane permeability (Jaenke, 1976; Olson et al., 1974; Arena et al., 1975).

Hemodynamic changes found during the development of congestive heart failure, directly correspond to the degree of cardiac dysfunction. Numerous studies were performed to evaluate acute and chronic effects of adriamycin on hemodynamic parameters reflecting cardiac pumping function and the peripheral vascular system.

17

Adriamycin cardiotoxicity was characterized by a progressive congestive heart failure that was refractory to inotropic agents and mechanical circulatory assistance (Lefrak et al., 1973). Patients with heart failure showed a marked fall in blood pressure, increase in heart rate, dilated ventricles and reduction in QRS voltage (Lefrak et al., 1973). Changes in the diastolic function have also been shown to occur early in the course of development of cardiomyopathy, even prior to any significant fall in the ejection fraction, stroke volume and cardiac index (Tomlinson, 1987). During the subsequent changes, a correlation between the change of the left ventricular shape and decrease in the ejection fraction, stroke volume and cardiac index was also noticed (Tomlinson, 1987).

F. SOME OTHER MAJOR ADRIAMYCIN SIDE EFFECTS

Although adriamycin-induced congestive heart failure is the most serious side effect, other tissues such as neuronal, renal, hematopoetic, etc., have also been shown to be effected.

F.1. Nephrotoxic Side Effects

A nephrotoxic syndrome was reported after the injection of 20 mg/kg i.v. of daunorubicin (Sternberg and Philips, 1967). Another study reported that 7.5 mg/kg of adriamycin (i.v.) caused injury to the glomerulus and made it more permeable to plasma proteins (Bizzi et al., 1983). Typical clinical symptoms of nephrotic syndrome including proteinuria, peripheral edema and massive hyperlipidaemia were observed. Ultrastructural glomerular studies revealed vacuolisation of the cytoplasm and thickening of the basal membrane (Sternberg, 1970; Young, 1975). Development of

18

chronic glomerulonephritis due to the nephrotoxicity of anthracyclins in rats as well as the presence of cytomegalic cells in renal tubular epithelium have also been reported (Sternberg et al., 1972). Using radioimmunoassay techniques, highest concentration of adriamycin equivalents among different tissues analyzed 24 hours after injection in rabbits was found in the kidney (Van Vunakis et al., 1974).

F.2. Hematotoxic Side Effects

Van Vleet et al., 1979, performed a study on pigs, giving them adriamycin i.v. in a dosage from 0.64 to 6.4 mg/kg/week for up to 20 weeks. Marked hypoplasia was seen in bone marrow and lymphoid tissue, with frequent terminal haemorrhagic diathesis and septicemia. Associated alterations in peripheral blood included leucopenia, anemia and thrombocytopenia.

F.3. Neurotoxicity

A neurotoxicity was reported in a number of experimental and clinical studies. Toxicity of central nervous system as well as peripheral neuropathies were described (Kondo et al., 1987; Gill et al., 1990). Kondo et al., (1987) have shown that after adriamycin treatment neuropathological changes due to the toxicity of the drug were found as early as day 4. The neurons in the cerebral cortex and nucleus caudatus-putamen showed focal clearing of the nuclear chromatin, increased dense bodies in the cytoplasm and dilatation of the cisternae of the rough endoplasmic reticulum and Golgi apparatus. By day 10, the cytoplasm of many neurons was vacuolated. During clinical studies, incidence of periferal neuropathy was found to be up to 80% (Willemse et al., 1990).

G. RAT AS AN ANIMAL MODEL

Characteristic myocardial cell structural changes associated with adriamycin-induced cardiomyopathy have been documented in humans (Lefrak et al., 1973; Buja et al., 1973; Jaenke, 1977; Bristow et al., 1978), which have also been noticed in a variety of animals such as rabbits (Jaenke, 1974; Olson et al., 1974), mice (Rosenhoff et al., 1975; Lambertenghi-Deliliers et al., 1976) and rats (Chalcroft et al., 1973; Singal et al., 1985). Some of the features noted in common included loss of myofibril, cytoplasmic vacuolization, swelling of mitochondria and increased number of lysosomes in humans and different animals models. Myofibrillar dropout and appearance of dilated tubular membrane vesicles have been used as the typical morphological mark of adriamycin cardiomyopathy. Thus rats seem to mimic many structural and functional features of adriamycin-cardiomyopathy in humans. Rat animal model has been used in a number of studies (Olson and Capen, 1977; Mettler et al., 1977; Zbinden et al., 1978; Siveski-Iliskovic et al., 1994). The model used in our study is highly reproducible and seems to be time, as well as dose-related, and comparable to changes seen in adriamycin-cardiomyopathy in humans (Deally and Singal, 1990; Siveski-Iliskovic et al., 1994). Sex related differences in adriamycin cardiomyopathy were not observed in patients (Von Hoff et al., 1979). Thus, with the animal model, generally male rats have been used.

H. SUGGESTED MECHANISMS OF ANTHRACYCLINE CARDIOTOXICITY

A large variety of subcellular effects of adriamycin have been reported and the list includes inhibition of DNA, RNA and protein synthesis; increase in number as

20

well as activation of lysosomes; changes of enzyme activity in mitochondria; alterations in membrane-bound enzymes; free radical formation and lipid peroxidation; impaired ion transport, calcium overload and effects of vasoactive substances. Since adriamycin is a lipophilic substance, a great variety of these adverse changes could be due to a direct membrane interaction of the drug. Alternatively, adriamycin-induced changes could be indirect through the production of oxygen free radicals (Singal et al., 1987). These direct and indirect changes may not be mutually exclusive and most probably, these cardiac effects and ultimately cardiomyopathy are a consequence of a combination of these different subcellular effects.

Characteristic features of adriamycin cardiomyopathy are a loss of myofibrils, possibly caused by its inhibition of nucleic acid and protein biosynthesis (Lefrak et al., 1973). An inhibitory effect of adriamycin on the formation of alpha-actinin/actin tridimensional networks and bundles has been reported (Dalle Donne et al., 1992). Some studies demonstrated that this mechanism is different from the mechanism responsible for adriamycin cytotoxicity. In this regard, vitamin E administration protected the heart against adriamycin cardiotoxicity without altering its antitumor properties (Myers et al., 1977). Increased lipid peroxidation and release of lysosomal enzymes may also contribute to adriamycin cardiotoxicity (Singal et al., 1985). Another ultrastructural hallmark is vacuolisation of the cytoplasm, which is due to a marked swelling of the sarcoplasmic reticulum and T-tubules (Billingham, 1979; Buja et al., 1973; Lefrak et al., 1973; Ferrans, 1978; Van Vleet et al., 1978; Singal,

21

1983), but the precise role of cytoplasmic vacuolisation in the myocardial function is still unclear. The sarcoplasmic reticulum is known to sequester intracellular calcium and is necessary for the muscle contraction-relaxation process. Interference with this process will affect myocardial contractility. Bellini et al., (1985) demonstrated that zinc iodide-osmium tetroxide (ZIO) reactive material could be seen in the sarcoplasmic reticulum soon after injection of adriamycin implying damage to the sarcoplasmic reticulum. A dose-related inhibition of Ca^{2+} uptake by adriamycin was observed in the rat heart sarcoplasmic reticulum preparation (Harris et al., 1985).

Adriamycin has been shown to influence the activity of a variety of sarcolemma-bound enzymes. In vitro experiments demonstrated that low concentrations of adriamycin stimulate adenylate cyclase activity and increase cyclic AMP concentration, while an inhibitory effect is noted at higher drug concentrations (Azuma et al., 1981; Singal and Panagia, 1984). There are conflicting reports concerning the effect of adriamycin on Na^+/K^+ ATPase activity (Gosalvez et al., 1979; Kim et al., 1980; Komori et al., 1985; Singal and Panagia, 1984). Ca^{2+} pump ATPase (Caroni et al., 1981) and 5^1-nucleotidase activity (Singal and Panagia, 1984) in the sarcolemma are not affected. These data imply that adriamycin may have selective effects on the functional activities of the sarcolemma.

Sarcolemmal Ca^{2+} bound at the low-affinity sites on the membrane has been associated with cellular Ca^{2+} influx and is correlated with the developed force (Bers et al., 1981; Dhalla et al., 1982). In in vitro studies, adriamycin was found to stimulate low-affinity Ca^{2+} binding but depress contractile function (Singal and

22

Pierce, 1986). Adriamycin in vitro also stimulates Ca^{2+} ATPase activity (Singal and

Panagia, 1984). These observations may indicate a permeability change leading to

an oversupply of Ca^{2+} due to increased Ca^{2+} influx. However, adriamycin-induced

oxidation of protein thiols and inhibition of Ca-ATPase activity have also been

reported (Vile and Winterbourn, 1990). Further evidence of this change is the

increase in LDH, CPK and SGOT enzymes in the serum of patients undergoing

adriamycin therapy (Lefrak et al., 1973). The intracellular localization of lanthanum,

an extracellular tracer, in the hearts of adriamycin-treated rats also indicated a

permeability change (Singal et al., 1985). During 30 min of perfusion of isolated

ferret hearts with adriamycin, Ca^{2+} significantly increased from a control, with

concomitant isovolumic left ventricular pressure decrease. This suggests that Ca

overload induced by adriamycin is associated with acute contractile failure (Kusuoka

et al., 1991).

Leakage of hydrolytic enzymes from the lysosome into the cell may be very

important in different pathological states. Studies demonstrated that lysosomal

changes are occurring in adriamycin treated animals (Singal et al., 1985). The

increased number of lysosomes and activated lysosomal hydrolysis in the myocardium

as well as in the circulation prior to an increased permeability of the sarcolemma

indicate that adriamycin has a direct effect on membrane system. These effects of

adriamycin on membrane systems are believed to be the consequence of the

formation of free radical and lipid peroxidation in the membrane. Myocardial

increase in MDA concentration, which is a product of lipid peroxidation, in

23

adriamycin-treated animals supports this hypothesis. In vitro study on adriamycin-induced leakage of lysosomal enzymes suggested that adriamycin had a direct effect on lysosomal membranes and may cause specific permeability changes leading to leakage of lysosomal enzymes. Superoxide dismutase (SOD) did not offer protection against these membrane changes, implying that the superoxide radical as such may not be contributing in the lipid peroxidation of lysosomal injury due to adriamycin (Singal and Tong, 1988).

Numerous studies have investigated functional changes in mitochondria. Calcium overload in mitochondria by ATP and respiration dependent mechanism was observed (Miwa et al., 1986). Adriamycin inhibits electron transport and oxidative phosphorylation and decreases mitochondrial ATP-ase activity. Decrease in myocardial high energy phosphates (HEP) in the myocardium treated with adriamycin have also been reported (Azuma et al., 1981; Muhamed et al., 1982; Jackson et al., 1983; Pelikan et al., 1986; Singal and Pierce, 1986). Depletion of myocardial ATP stores, can be due to lack of production and/or increased consumption accompanied by stimulation of Ca^{2+}-activated enzyme activities under the influence of intracellular Ca^{2+} overload (Revis and Marusic, 1979; Singal et al., 1985; Dhalla et al., 1985). Hexakis (2-methoxyisobutyl isonitrile) technetium I, a gamma-emitting radiopharmaceutical with myocellular accumulation properties dependent on mitochondrial membrane potential, was used for monitoring adriamycin-induced alterations in cardiac energetics. Adriamycin concentrations as low as 0.1 μM inhibited its accumulation within mitochondria (Piwnica-Worm et al.,

24

1993). Studies of mitochondrial lipid peroxidation, membrane fluidity and activities of mitochondrial respiratory chain in isolated mice mitochondria 48 hours after a single adriamycin injection did not show any significant acute toxicity. However, mitochondrial toxicity was detected after drug was given chronically (Praet and Ruysschaert, 1993). Specific binding of adriamycin to the phospholipid cardiolipin is related to the inhibition of mitochondrial activity (Nicolay et al., 1987). The low energy state in cardiac tissue was demonstrated by ^{31}P-NMR measurements in the living animal (Nicolay et al., 1987). Adriamycin also caused breakage of the mitochondrial DNA helix and slowed the rate of mitochondrial DNA synthesis and the formation of complete DNA molecules (Ellis et al., 1987).

There is a good correlation between the severity of adriamycin-induced cardiac failure and impairment of mitochondrial function (Bachman and Zbinden, 1975, 1979).

Another suggested mechanism of adriamycin cardiomyopathy is through adriamycin-induced production of heat-shock proteins, which then stimulate potent T lymphocyte responses that may contribute to cardiac damage (Huber, 1992). In in vivo studies, it was demonstrated that adriamycin treated mice developed both cytolytic T lymphocytes and antibodies to drug-treated myocytes (Huber and Moraska, 1992). Adriamycin selectively decreased alpha cardiac actin mRNA in the rat heart when compared to other mRNAs examined in the heart and skeletal muscle, and administration of cardioprotective chelating agent ICRF-187 did not modify this effect (Papoian and Lewis, 1990, 1991). This may suggest that decrease

in actin mRNA was not related to iron mediated free radical formation, but some other mechanisms.

I. FREE RADICALS AND LIPID PEROXIDATION

Free radical is a molecule or atom that contains one or more unpaired electrons and is highly reactive. It can act as an oxidizing and/or reducing agent depending upon the substrate. Production of free radicals generally has local effect. However, secondary radicals, formed from them, can also have distant biological effects via a chain reactions.

Adriamycin has been shown to be a potential source of free radicals. In the presence of oxygen, the adriamycin semiquinone radical can react to yield superoxide radical. The superoxide dismutase can dismutate superoxide radical to form hydrogen peroxide and subsequently interacts with this product to form the hydroxyl radical. The superoxide radical, hydrogen peroxide and hydroxyl radical, thus produced, can initiate a chain reaction which results in oxidative damage of the cell membrane. Peroxidation of polyunsaturated fatty acids in the cell membrane leads to severe damage and degradation of the cell membrane and protein aggregation. Hydroxyl radical formed can react with polyunsaturated fatty acids to form lipid radicals and lipid hydroperoxide. In the presence of transition metal ions, the chain reaction continues. It has been suggested that iron plays an important role in adriamycin induced lipid peroxidation. Without iron, the amount of MDA produced by adriamycin is small and even a low concentration of iron can stimulate its production (Griffin-Green et al., 1988). In iron mediated reactions, adriamycin can

26

be reduced enzymatically by NADH dehydrogenase to semiquinone radical, which in turn can react with molecular oxygen to start inorganic and organic radical chain reactions.

It is possible that adriamycin transfers an electron directly to Fe^{3+}, after which the generated Fe^{2+} can reduce oxygen to hydrogen peroxide (Hasinoff et al., 1988). This reaction was shown to occur for the adriamycin-Fe^{3+} complex, which also produces free radicals (Teicher et al., 1988). The Fe^{3+}-adriamycin complex binds cytochrome c oxidase, the terminal respiratory enzyme and inhibits its function (Hasinoff et al., 1988). The Fe^{3+}-adriamycin-cytochrome c oxidase complex initiates free radical reaction, OH could damage the nearby cardiolipin bound to cytochrome c oxidase (Hasinoff et al., 1988). ICRF-187, an iron chelator has been reported to moderate adriamycin cardiomyopathic changes (Herman and Ferrans, 1981).

The potential targets of lipid peroxidation are all membrane systems which contain polyunsaturated fatty acids. Cytofluorescence techniques have revealed that specific binding of adriamycin to mitochondria occurs in rat heart (Nicolay et al., 1984) and adriamycin appears to have a high affinity for the negatively-charged phospholipid, cardiolipin, which is abundant in the inner membrane of mitochondria (Goormaghtigh et al., 1980, 1986; Nicolay et al., 1984, 1985). In an in vivo study, adriamycin caused significant increases in cardiac lipid peroxides but, no lipid peroxidation was observed in the liver. Possible explanation for this observation could be low levels of glutathione peroxidase activity in the heart and high levels of the same antioxidant enzyme in the liver (Odom et al., 1992) Although the details

of the processes of lipid peroxidation are not clear, the measurement of other end products such as propane, ethane and pentane can also provide indication of the formation of lipid peroxide (Hunter et al, 1964). The fatty acid content of the membrane is also used to determine the extent of lipid peroxidation (Griffin-Green et al., 1988). Several investigators have calculated that 1 mole of MDA is generated for every 14-21 moles of unsaturated fatty acids consumed (Baker et al., 1966; Reiter et al., 1987; May et al., 1968).

Increased MDA levels were reported in both in vitro and in vivo studies examining the myocardial effects of adriamycin (Singal et al., 1986; Myers et al., 1977). Reduction of adriamycin cardiotoxicity by the use of different antioxidants provide support to the hypothesis that adriamycin cardiomyopathy may be mediated by lipid peroxidation (Pascoe et al., 1987; Tanigawa et al., 1986; Myers et al., 1977). In addition to the indirect approach of measurements of end products, increased levels of oxygen species due to adriamycin have also been detected directly by electron spin resonance spectroscopy (Costa et al., 1988; Thornalley et al., 1985; Alegria et al., 1989). Thus, free radical and lipid peroxidation are recognized as one possible mechanism of adriamycin cardiotoxicity.

J. ANTIOXIDANTS AND ADRIAMYCIN CARDIOTOXICITY

There is a very fine balance between cellular systems that generate various oxidants and those that maintain antioxidant defense mechanisms (Singal and Kirshenbaum, 1990). Adriamycin, by its free oxygen radicals generating properties, can tip this balance in favour of oxidants. Endogenous antioxidants like superoxide

28

dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) are no longer able to cope with this increase in free radical production and adriamycin cardiotoxicity gets expressed. Since the late 1970's when free oxygen radicals were identified as a factor responsible for adriamycin-induced cardiomyopathy, researchers have been trying to ameliorate or block this negative side effect by employment of different endogenous as well as exogenous antioxidants.

Myers et al., (1977), demonstrated that intraperitoneal injection of α-tocopherol (vitamin E) can ameliorate acute toxic effects of adriamycin if given 24 hours prior to adriamycin administration. However, further studies demonstrated that α-tocopherol is effective against acute adriamycin cardiotoxicity in mice (up to 15 days after adriamycin administration) while it had no significant effect on adriamycin lethal toxicity after 9 weeks of administration, which suggests that α-tocopherol delayed rather than prevented adriamycin lethal toxicity in mice (Mimnaugh et al., 1979).

In another study on dogs, no cardioprotection against chronic adriamycin cardiomyopathy by vitamin E and selenium was seen (Van Vleet et al., 1980). Similarly, no significant protective action with vitamin E and/or selenium against adriamycin induced delayed lethality was seen in mice (Hermansen and Wassermann, 1985). However, vitamin E deficient diet in rats resulted in significantly higher mortality as well as more ultrastructural myocardial damage due to adriamycin (Singal and Tong, 1988).

Administration of ascorbic acid, a strong antioxidant, to the mice inoculated

with leukemia L1210 or Ehrlich ascites carcinoma had no effect on the antitumor activity of adriamycin but significantly prolonged the life of animals treated with adriamycin. Ultrastructural changes due to adriamycin cardiomyopathy were also significantly ameliorated (Shimpo et al., 1991).

In 1981, Herman and Ferrans pretreated dogs with ICRF-187 (NSC 169780) 30 min before giving doxorubicin (adriamycin) (12.5 mg/kg, weekly). After the 15th injection in dogs that got doxorubicin alone, they noted vacuolization and myofibrillar loss in myocardium, using light microscopy. In contrast, minimal abnormalities were noticed in the hearts of dogs given ICRF-187 before doxorubicin administration. ICRF-187 is reacting directly with iron and increasing its excretion, hence reducing free oxygen radical production through Fenton reaction. Another study demonstrated that cardioprotective action of ICRF-187 during adriamycin treatment may be a result of its hydrolysis to the d isomer of ICRF-198, which inhibits reduction of Fe^{3+}, thus limiting the role of iron in tissue damaging free radical reactions (Vile and Winterbourn, 1990). Unfortunately, the addition of ICRF-187 to the adriamycin-containing treatment is increasing the hematological toxicity. Hence, any clinical application of ICRF-187 is limited because of the above mentioned characteristic feature. Paracchini et al., (1993), demonstrated that a spin trapping compound alpha-phenyl-tert-butyl nitrone (PBN), given concurrently with adriamycin, improved ECG and prevented the myelotoxicity of adriamycin without any negative effect of adriamycin cytotoxicity evaluated in vitro (Paracchini et al., 1993). Oleanolic acid and Ursolic acid, both components with free radical scavenging

potential have shown protection against adriamycin induced lipid peroxidation studies in liver and heart microsomes in vitro (Balanehru and Nagarajan, 1992). A number of newly synthesized substances have also been tested in an attempt to achieve cardioprotection against adriamycin cardiotoxicity. PZ51(Ebselen), a novel selenoorganic compound offered some protection against adriamycin-induced lipid peroxidation in heart and liver tissue and adriamycin-induced toxicity in general, as measured by total serum creatine kinase activity and body weight in the mouse (Pristos et al., 1992). Thus, major effort is needed in studying potential pharmacological substances that would decrease and/or prevent adriamycin cardiotoxicity with no concomitant decrease in its antineoplastic activity.

K. PROBUCOL AS AN ANTIOXIDANT

Probucol was first introduced in the early 1970's as a lipid lowering drug. Though it was used because of its LDL-cholesterol-lowering properties, it was soon noted that the drug lowered HDL-cholesterol more than it lowered LDL-cholesterol. However, in the 1980's, it became clear that probucol has antioxidant properties as well. Probucol treatment in heterozygous familial hypercholesterolemia caused the regression of xanthomas which did not correlate with the level of cholesterol reduction (Yamamoto et al., 1986). Cholestyramine, another cholesterol lowering drug, and probucol, both sharply lowered the serum cholesterol levels in nonhuman primates while only probucol caused regression of atherosclerotic lesions (Wissler et al., 1983). These observations clearly suggest that the beneficial effects of probucol may be independent of cholesterol lowering effect. Probucol has no apparent

31

structural similarity to other agents that lower cholesterol concentrations. It is a bis-phenol which resembles vitamin E, another well known antioxidant but with only one phenolic group. Because of the two phenolic groups in its molecular structure, probucol has been reported to be a strong antioxidant (Mao et al., 1991; Pryor et al., 1988) and that opened a whole new perspective for this drug. Vitamin E radical produced during the radical quenching process is reoxidized by vitamin C. Thus vitamin E and C compliment each other. Similar reaction for probucol has not been reported thus far. In this study we have examined whether probucol, with its already demonstrated antioxidant properties, have beneficial effects against the development of adriamycin-induced cardiomyopathy and consequent congestive heart failure.

IV. MATERIALS AND METHODS

A. ANIMAL MODEL AND TREATMENT PROTOCOLS

Male Sprague-Dawley rats, body weight 250 ± 10 g, were maintained on normal rat chow on normal light and dark cycle. Animals were given water and food ad libitum. For studying the beneficial effects of probucol, two different approaches were adopted for treatment of the animals. In the first approach, adriamycin and probucol treatments were given on alternate days starting from day one (concurrent therapy). In the second approach, probucol treatment alone was started 2 weeks prior to the initiation of concurrent therapy.

In the concurrent therapy part, rats were divided in four groups: CONT (control), ADR (adriamycin treated), PROB (probucol treated) and PROB + ADR (probucol + adriamycin treated). Adriamycin (doxorubicin hydrochloride) was administered intraperitoneally (Monday, Wednesday and Friday) in six equal injections (each containing 2.5 mg/kg ADR) to animals in ADR and PROB + ADR groups over a period of 2 weeks for a cumulative dose of 15 mg/kg body weight. Probucol (cumulative dose, 60 mg/kg body weight) was also administered intraperitoneally to PROB and PROB + ADR groups in six equal injections (each treatment containing 10 mg/kg) over a period of 2 weeks, alternating with adriamycin injections (Tuesday, Thursday and Saturday). CONT animals were injected with the vehicle alone (lactose, 75 mg/kg in saline) in the same regimen as ADR. For a pretreatment with probucol, animal groupings were done as described above. Probucol injections (each containing 10 mg/kg) were given over a period of two

33

weeks prior to the concurrent treatment with adriamycin and probucol.

B. GENERAL OBSERVATIONS

Animals were monitored daily during the treatment as well as post-treatment periods for body weight, general appearance, behaviour and mortality. At the end of the 3 week post-treatment period, animals were hemodynamically assessed. Hearts were excised, weighed and used for the study of myocardial antioxidants, lipid peroxidation and ultrastructure. The amount of ascites (fluid in the peritoneal cavity) was measured using aspiration method in which a 60 ml syringe was employed.

C. HEMODYNAMIC STUDIES

Animals were anesthetized by sodium pentobarbital (50 mg/kg, i.p.). A miniature pressure transducer (Miller Micro-Tip) was inserted into the left ventricle via the right carotid artery. Left ventricular systolic pressure (LVSP) and end-diastolic pressure (LVEDP) as well as aortic systolic (ASP) and diastolic pressures (ADP) were recorded on a Beckman Dynograph recorder and on a computer for on-line analysis (Axotape acquisition data program). After catheterization, animals were allowed to stabilize for at least 15 minutes prior to the recording of hemodynamic data.

D. BIOCHEMICAL STUDIES

D.1. Catalase

Ventricles were homogenized in 9 vol. of 0.05M potassium phosphate buffer (pH 7.4) and centrifuged at 40,000 g for 30 min. Supernatant, 50 μL, was added to the cuvette containing 2.95 ml of 19 mM H_2O_2 solution prepared in potassium

34

phosphate buffer (Claiborne, 1985). The colour was read at 240 nm on a Spectronic 601 spectrophotometer every min for 5 min. Commercially available catalase was used as a standard. Specific activity of the enzyme was expressed as units per milligram tissue protein.

D.2. Glutathione Peroxidase (GSHPx)

GSHPx activity was expressed as nanomoles of reduced nicotinamide adenine dinucleotide phosphate (NADPH) converted to oxidized nicotinamide adenine dinucleotide phosphate (NADP) per min per milligram protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22 x 10^6 (Paglia and Valentine, 1967). Cytosolic GSHPx was assayed in a 3 ml cuvette containing 2.0 ml of 75 mM phosphate buffer, pH 7.0. The following solutions were then added: 50 μl of 60 mM glutathione, 100 μl glutathione reductase solution (30 U/ml), 50 μl of 0.12 M Na N_3, 100μl of 15 mM Na_2 EDTA, 100 μl of 3.0 mM NADPH and 100 μl of cytosolic fraction obtained after centrifugation at 20,000 g for 25 min. Water was added to make a total volume of 2.9 ml. The reaction was started by the addition of 100 μl of 7.5 mM H_2O_2, and the conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-min intervals for 5 min. Enzyme activity of GSHPx was expressed in terms of milligrams of protein.

D.3. Superoxide Dismutase (SOD)

Supernatant (20,000 g for 20 min) was assayed for SOD activity by following the inhibition of pyrogallol autooxidation (Marklund, 1985). Pyrogallol (24 mM) was prepared in 10 mM HCl and kept at 4°C before use. Catalase (30 μM stock

solution) was prepared in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 μg protein) were added to Tris HCl buffer containing 25 μl pyrogallol and 10 μl catalase. The final volume of 3 ml was made up with the same buffer. Changes in absorbance at 420 nm were recorded at 1-min intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autooxidation with a known SOD activity. This assay was highly reproducible, and the standard curve was linear up to 250 μg protein with a correlation coefficient of 0.998. Data were expressed as SOD units per milligram protein as compared with the standard.

D.4. Malondialdehyde

Measurement of lipid peroxidation by determining myocardial malondialdehyde (MDA) content was performed using a modified thiobarbituric acid (TBA) method (Placer et al., 1966). Hearts were quickly excised and washed in buffered 0.9% KCl (pH 7.4). After removing the atria, extraneous fat and connective tissue, the ventricles were homogenized (in 10% w/v) in the same buffer. The homogenate was incubated for 1 hour at 37°C in a water bath. A 2-mL aliquot was withdrawn from the incubation mixture and pipetted into an 8-mL Pyrex tube. One millilitre of 40% trichloroacetic (TCA) acid and 1 mL of 0.2% TBA were promptly added. To minimize peroxidation during the subsequent assay procedure, 2% butylated hydroxytoluene was added to the TBA reagent mixture (Aust, 1985). Tube contents were vortexed briefly, boiled for 15 min and cooled in a bucket of ice for 5 min. Two millilitres of 70% TCA was then added to all tubes and contents were

36

again vortexed briefly. The tubes were allowed to stand for 20 min. This was followed by a centrifugation of the tubes for 20 min at 3500 rpm. The colour was read at 532 nm on a Spectronic 601 spectrophotometer and compared with a known MDA standard.

E.   ULTRASTRUCTURAL STUDIES

For ultrastructural studies, hearts were processed as described (Tong et al., 1991; Singal et al., 1985). Hearts were washed in cold 0.1 M sodium phosphate buffer (pH 7.4). Tissue samples, 4-6 mm in size were taken from four different areas of the subendocardium, as well as subepicardium of the free left ventricle wall between the mid-region and apex of the heart. The tissue pieces were immersed for 15 min in 0.1 M phosphate buffer (pH 7.4) containing 3% glutaraldehyde. This briefly fixed tissue was further cut into pieces smaller than 1-mm cubes. Aldehyde fixation was continued for a total duration of 2 hours. The tissues were washed for 1 hour in the above phosphate buffer containing 0.05 M sucrose. Postfixation was done in 2% OsO_4 for 1.5 hours, after which the tissue pieces were dehydrated in graded alcohol series. Tissue embedding was done in epon. Ultrathin sections were placed on Formvar coated grids and stained with uranyl acetate and lead citrate. Electron micrographs of the subendocardial and subepicardial regions from the four groups were compared to establish ultrastructural differences.

F.   PROTEIN DETERMINATION AND STATISTICAL ANALYSIS

Proteins were determined by the method of Lowry and associates (1951). Data were expressed as the mean ± SEM. For a statistical analysis of the data,

group means were compared by one-way analysis of variance and Bonferroni's test was used to identify differences between groups. Statistical significance was acceptable to a level of P <0.05.

V. RESULTS

A. GENERAL OBSERVATIONS AND MORTALITY

The general appearance of animals in all groups was recorded during the course of study. In ADR as well as PROB + ADR (concomitant treatment) groups, the animal fur became scruffy and developed a light yellow tinge. In these animals, there was red exudate around the eyes. These changes were more extensive in the ADR group. Animals in ADR groups also appeared to be more sick, weak and lethargic when compared with the PROB + ADR group. Animals in the CONT and PROB groups appeared normal.

The most predominant feature in the ADR group of animals was the development of a grossly enlarged abdomen and presence of ascites. This condition became apparent within one week after the completion of treatment with adriamycin. In a quantitative analysis, ascites in the ADR group was found to be 92.2 ± 13.2 ml, whereas probucol treatment in the PROB + ADR group reduced the formation of ascites to 19.9 ± 6.4 (Table 1). During the three-week post-treatment period, mortality rate was significantly higher in the ADR group, than in the PROB + ADR group (Table 1). There were no deaths or ascites in the CONT and PROB groups (Table 1). At the time of sacrifice, the ADR treated animals not only had a significant amount of fluid in the abdominal cavity but they also showed enlarged livers.

In order to test if increased exposure to probucol would result in improved protection, in a follow up study, animals in PROB + ADR group were first

pretreated with the probucol for two weeks and then concomitantly treated with probucol and adriamycin for the next two weeks. Data from these experiments are shown in Table 2. After completion of adriamycin treatment, only five animals in PROB + ADR group developed a light yellow tinge on their fur. During the post-treatment period, mortality rate in the ADR group was comparable to as seen before. However, probucol in these experiments offered a complete protection with respect to ascites as well as liver congestion. Out of 14 animals examined for ascites in PROB and ADR group, 12 animals had no ascites, 1 animal had 6 ml and 1 animal had 13 ml of ascites.

B. BODY WEIGHT

The most predominant feature in the treated animals was their weight loss during the treatment which showed a small recovery during the post treatment period (Fig 1). The weight loss due to adriamycin, became apparent after two injections and there was significant difference between the control and treated animals throughout the post-treatment duration (Figure 1). During the post-treatment period, there was a significant recovery in body weight in the ADR and PROB + ADR groups. Probucol treatment did not influence any body weight changes in any of the groups (Fig 1).

During the pre and concurrent treatment durations, probucol had no effect on the body weight gain in any of the groups (Fig 2). An increased weight gain in ADR groups during the post treatment period in these experiments corresponded to the increased ascites noted in this group (Table 2) as compared to that seen in the

ADR group in Table 1.

C. <u>HEART WEIGHT AND RATIO WITH THE BODY WEIGHT</u>

Treatment with adriamycin resulted in a significant decrease in the heart weight and heart to body weight ratio in the ADR group compared to CONT, PROB and PROB + ADR groups (Tables 1 and 2). Probucol treatment in the PROB + ADR group, resulted in significantly improved heart weight but it was still below the control levels (Table 1). In the pretreatment group, probucol resulted in a complete recovery of heart weight. In the PROB + ADR group, heart to body weight ratio was not significantly different from the CONT and PROB groups (Table 1 and 2).

TABLE 1: EFFECTS OF PROBUCOL (CONCURRENT) TREATMENT ON ADRIAMYCIN-INDUCED CHANGES ON HEART WEIGHT, BODY WEIGHT, MORTALITY AND ASCITES IN RATS.

Animal Group	Heart weight (g)	Heart weight/ Body weight ratio x 10^3	Mortality (%)	Ascites (ml)
CONT	1.25 ± 0.05	2.84 ± 0.12	0	0
ADR	0.75 ± 0.03*	2.38 ± 0.08*	30	92.2 ± 13.2*
PROB	1.18 ± 0.04	2.78 ± 0.13	0	0
PROB + ADR	0.92 ± 0.04†	2.73 ± 0.14	10	19.9 ± 6.4†

CONT, control; ADR, adriamycin; PROB, probucol and PROB+ADR, probucol and adriamycin. Data are mean ± S.E. of 6-8 animals in all studies. Mortality data are mean ± S.E. of 50 animals each in the ADR and PROB+ADR groups and 25 animals each in the CONT and PROB groups. * and †) P< 0.05 as compared to all other groups.

42

TABLE 2: EFFECTS OF PROBUCOL (PRE & CONCURRENT) TREATMENT ON ADRIAMYCIN-INDUCED CHANGES IN HEART WEIGHT, BODY WEIGHT, MORTALITY RATE AND ASCITES.

Animal Group	Heart weight (g)	Heart weight/ Body weight ratio x 10^3	Mortality (%)	Ascites (ml)
CONT	1.27 ± 0.04	2.80 ± 0.10	0	0
ADR	0.76 ± 0.03*	2.22 ± 0.12*	32	105.2 ± 19.3*
PROB	1.20 ± 0.06	2.65 ± 0.08	0	0
PROB + ADR	1.04 ± 0.04	2.71 ± 0.15	0	1.35 ± 0.99

CONT, control; ADR, adriamycin; PROB, probucol; and PROB+ADR, probucol and adriamycin. In these experiments probucol treatment was started 2 weeks prior to and was continued for another 2 weeks concurrently with adriamycin treatment. Data are mean ± S.E. of 6-8 animals in all studies. Mortality data are mean ± S.E. of 25 animals each in the ADR and PROB+ADR groups and 12 animals each in the CONT and PROB groups. *) P< 0.05 as compared to all other groups.
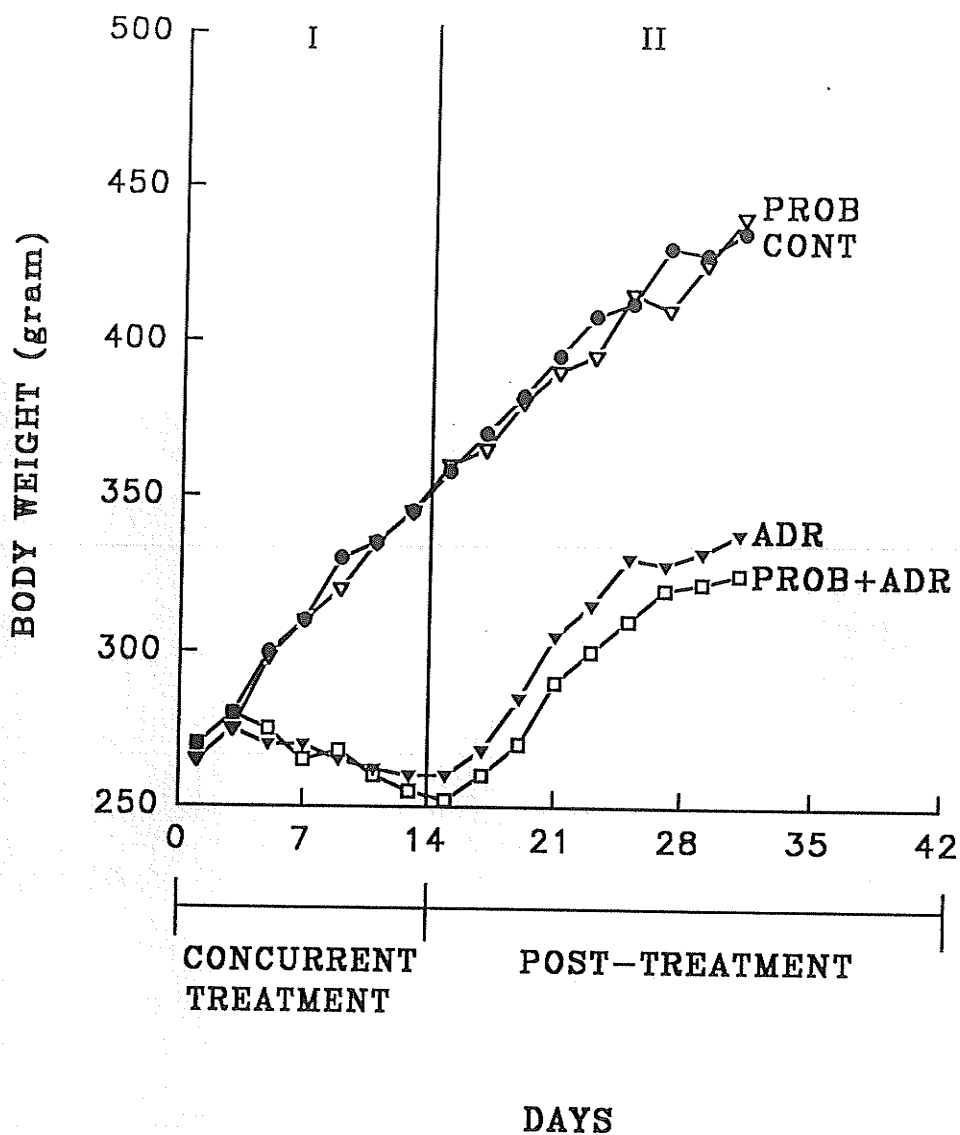
Figure 1: Effects of adriamycin (ADR), probucol (PROB) and concurrent probucol and adriamycin (PROB + ADR) treatment on body weight in rats. I. Two week treatment period. II. Post-treatment duration. Mean of 50 animals each in ADR and PROB + ADR groups and 25 animals each in CONT and PROB groups. S.D. not shown for keeping clarity. Both ADR and PROB + ADR groups daily weight loss was significant.

44
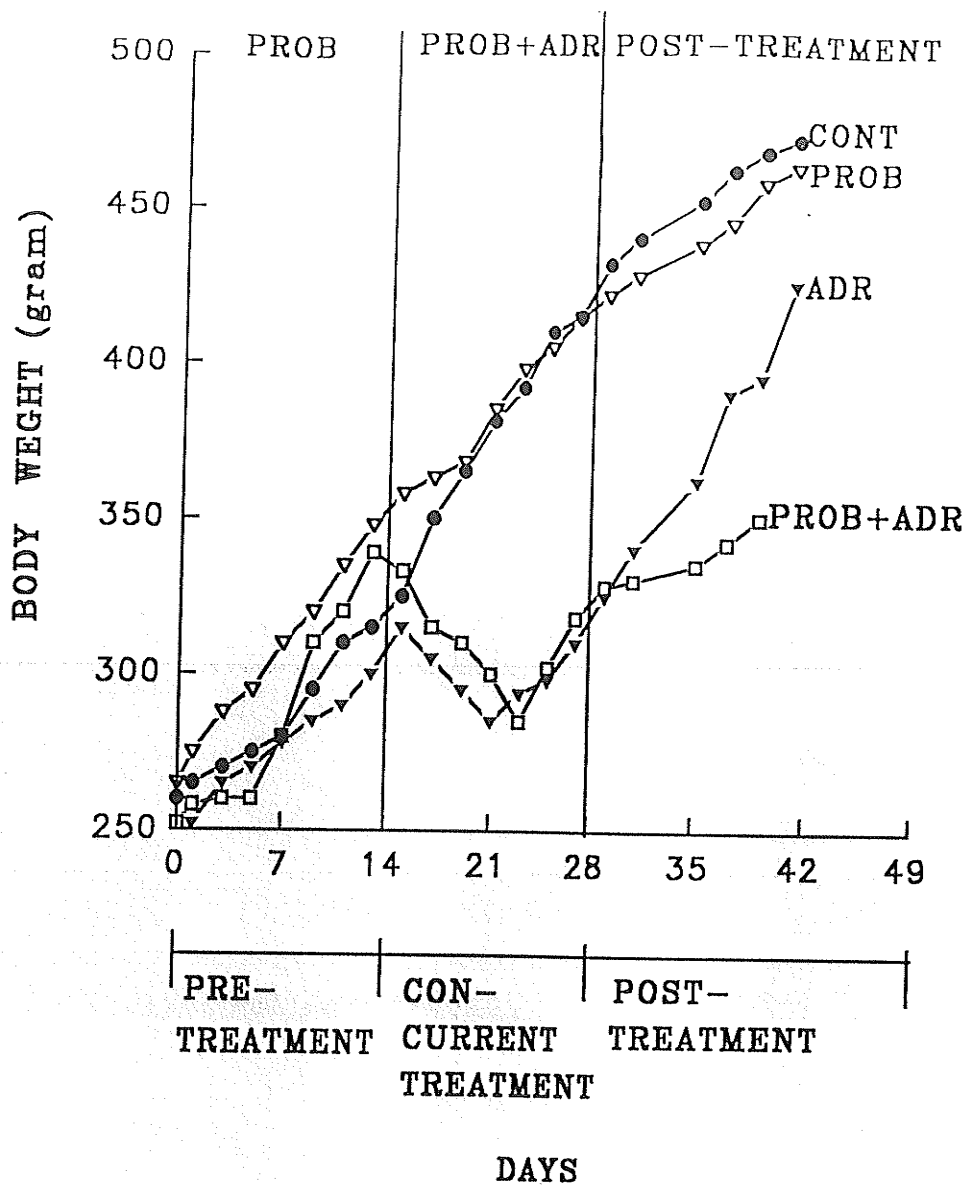
Figure 2: Effects of probucol pre and concurrent treatment with adriamycin (PROB + ADR) on body weight in rats. PROB; two week pretreatment period with probucol. PROB + ADR; two week concurrent probucol and adriamycin treatment. POST-TREATMENT; three weeks post-treatment duration. Mean of 50 animals each in ADR and PROB + ADR groups and 25 animals each in CONT and PROB groups. S.D. not shown for keeping clarity. In both ADR and PROB + ADR groups, body weight loss was significant.

D. HEMODYNAMIC STUDIES

Cardiac function as well as blood pressure readings were taken by placing a catheter with a micro-tip pressure transducer first in the aorta and then advancing into the left ventricle. These data on aortic systolic (ASP) and diastolic (ADP), left ventricular peak systolic (LVPSP) and end diastolic (LVEDP) pressures in all groups are shown in Table 3 for the concurrent treatment study and in Table 4 for the pre as well as concurrent treatment study.

There were significant changes in cardiac performance in ADR group (Table 3). LVPSP and ASP were both significantly depressed, while LVEDP was significantly elevated. Probucol treatment in the PROB + ADR group maintained ASP as well as LVPSP. The LVEDP, though it showed some improvement, was still higher than CONT and PROB groups. There was no difference between CONT and treated groups with respect to ADP (Table 3).

In the study on pretreatment with probucol (Table 4), all values in the PROB + ADR group were maintained at the control level. This was also true for LVEDP. Probucol treatment by itself had not effect on any of the parameters examined in these studies.

E. MORPHOLOGICAL STUDIES

Electron microscopic analysis of left ventricular free wall was conducted on heart tissue from all four groups of rats. The morphological appearance of different subcellular structures including mitochondria, sarcoplasmic reticulum, sarcomeres, myofibrils and intercalated discs in hearts from control and probucol groups were

46

TABLE 3: EFFECTS OF PROBUCOL (CONCURRENT) TREATMENT ON ADRIAMYCIN-INDUCED PRESSURE CHANGES (mmHG).

Animal Group	ASP	ADP	LVPSP	LVEDP
CONT	102.1 ± 1.8	66.9 ± 4.4	126.0 ± 11.3	5.9 ± 3.0
ADR	84.2 ± 3.2*	57.8 ± 9.4	89.5 ± 6.7*	33.7 ± 8.6*
PROB	91.0 ± 9.8	62.6 ± 13.5	113.9 ± 6.5	12.4 ± 6
PROB + ADR	110.5 ± 8.2	72.1 ± 6.3	123.3 ± 7.2	20.1 ± 5.3†

Values are mean ± S.E. of 6-8 experiments. * and †) $P < 0.05$ significantly different from CONT and ADR groups. CON, control; ADR, adriamycin; PROB, probucol; PROB + ADR, probucol and adriamycin; ASP, aortic systolic pressure; ADP, aortic diastolic pressure; LVPSP, left ventricular peak systolic pressure; LVEDP, left ventricular end diastolic pressure.

TABLE 4: EFFECTS OF PROBUCOL (PRE & CONCURRENT) TREATMENT ON ADRIAMYCIN-INDUCED PRESSURE CHANGES (mmHG).

Animal Group	ASP	ADP	LVSP	LVEDP
CONT	103.2 ± 2.7	66.6 ± 3.4	124.5 ± 1.3	5.3 ± 2.6
ADR	83.3 ± 4.5*	60.3 ± 8.2	86.5 ± 5.8*	35.6 ± 4.9*
PROB	94.3 ± 4.3	58.6 ± 6.5	117.2 ± 2.7	7.4 ± 3.6
PROB + ADR	104.0 ± 8.7	62.6 ± 3.3	112.22 ± 9.5	9.1 ± 1.3

Values are mean ± S.E. of 6-8 experiments. *) $P<0.05$ significantly different from CONT, PROB and PROB + ADR groups. CON, control; ADR, adriamycin; PROB, probucol; PROB + ADR, probucol and adriamycin; ASP, aortic systolic pressure; ADP, aortic diastolic pressure; LVPSP, left ventricular peak systolic pressure; LVEDP, left ventricular end diastolic pressure.

typical of normal cells (Fig 3). Morphological changes due to ADR treatment alone included disruption of several subcellular elements including loss of myofibrils, swelling of mitochondria, vacuolisation of the cytoplasm, formation of lysosomal bodies and dilation of the sarcotubular system (Fig 4). Mitochondrial injury in addition to the swelling of these organelles was also accompanied by disarrangement and disruption of cristae (Fig 5). Some of the electron dense bodies showed lamellar inclusions (Fig 5).

Ultrastructure of myocytes in the PROB + ADR group, from the concomitant-treatment study, showed regular myofibrilar structure, maintained sarcotubular reticulum and preserved mitochondria (Fig 6). At higher magnification, intramitochondrial details were quite normal but some intracellular edema was noticeable around the mitochondria (Fig 7).

In the pre as well as concomitant probucol treatment study, ultrastructure of myocytes from the PROB and ADR group showed a complete protection of the subcellular details (Figs 8 and 9).

FIGURE 3:

Portion of a myocardial cell from a control rat: mitochondria (M); myofibrils (MF); sarcoplasmic reticulum (arrows). X 25,000
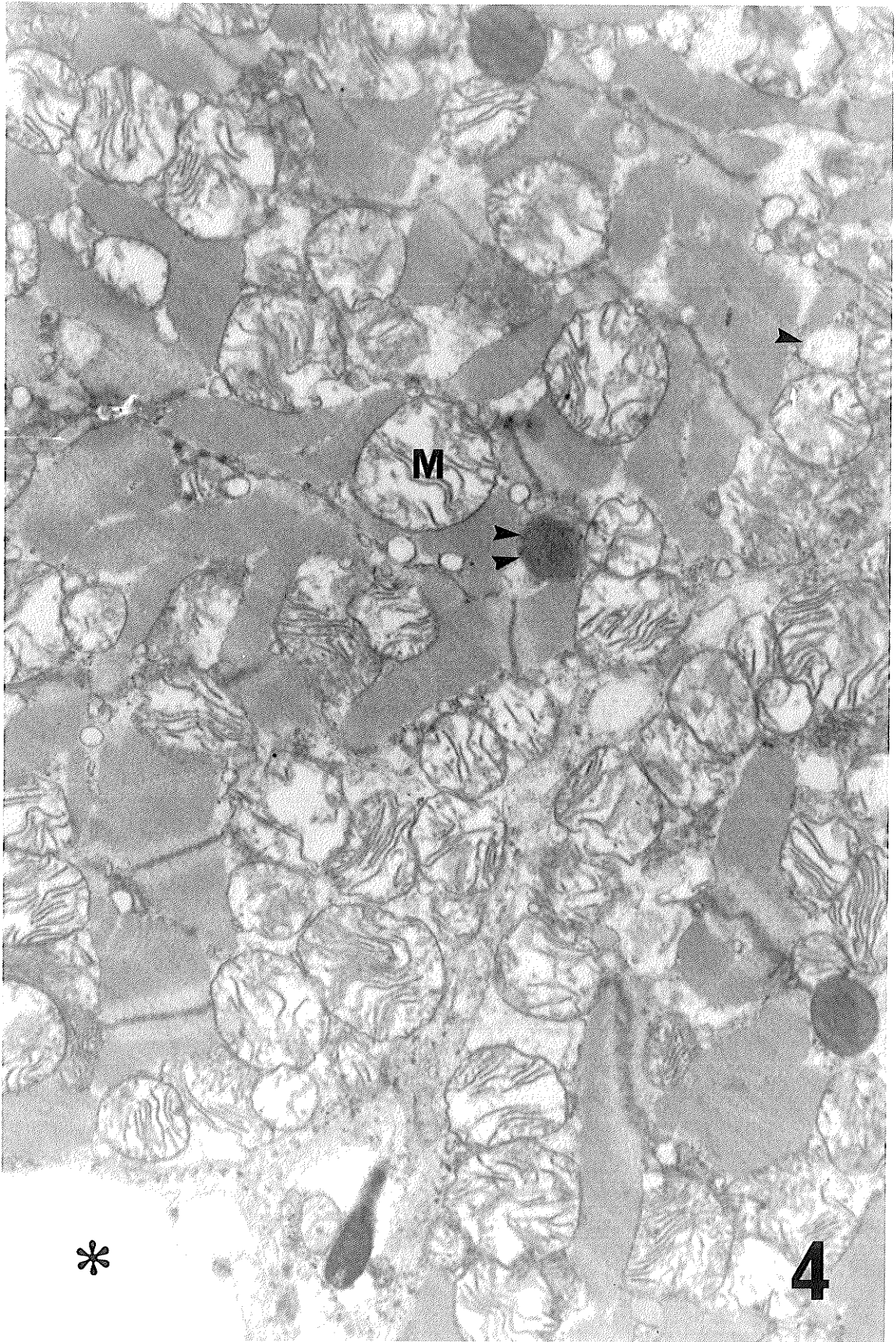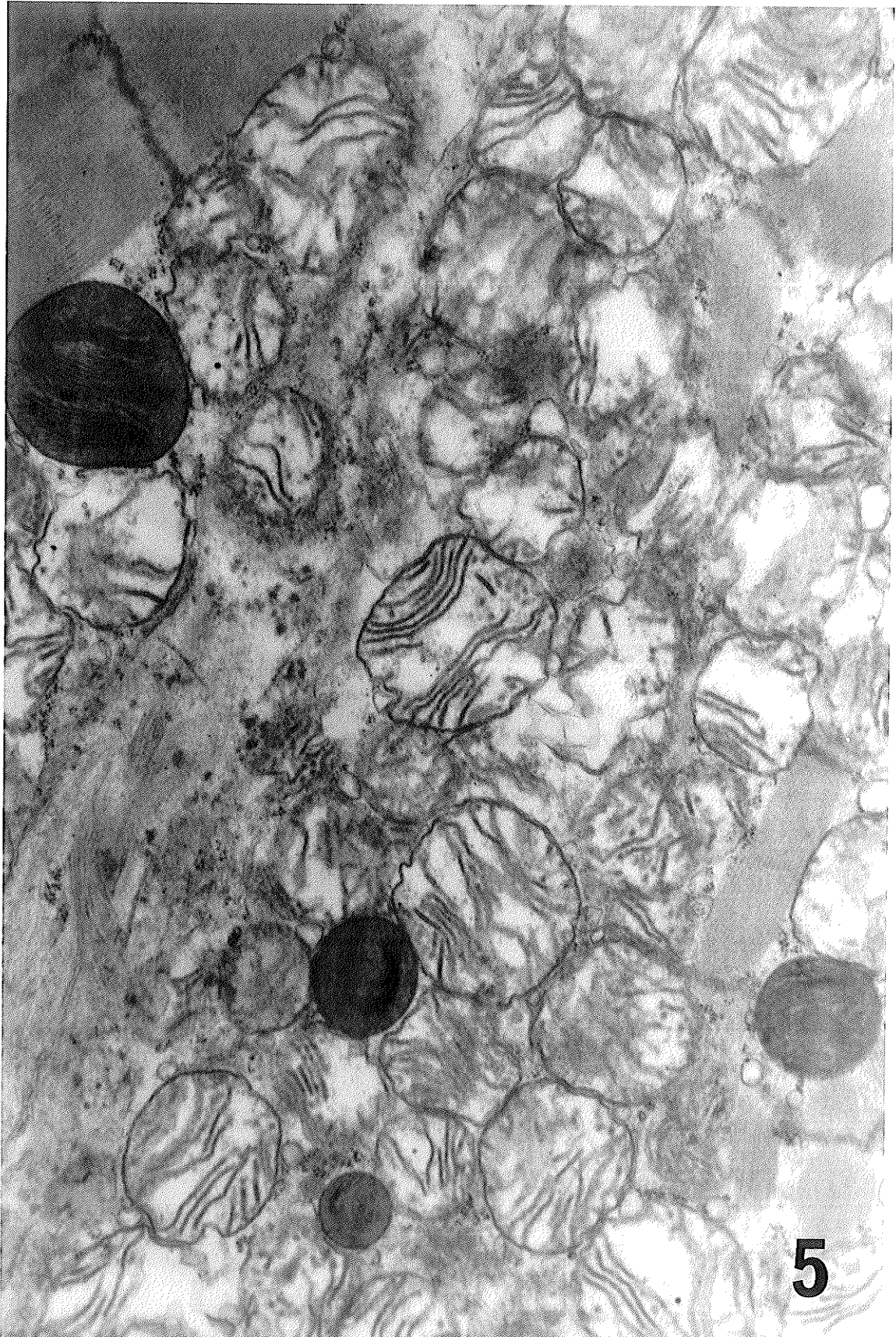
M

MF

3
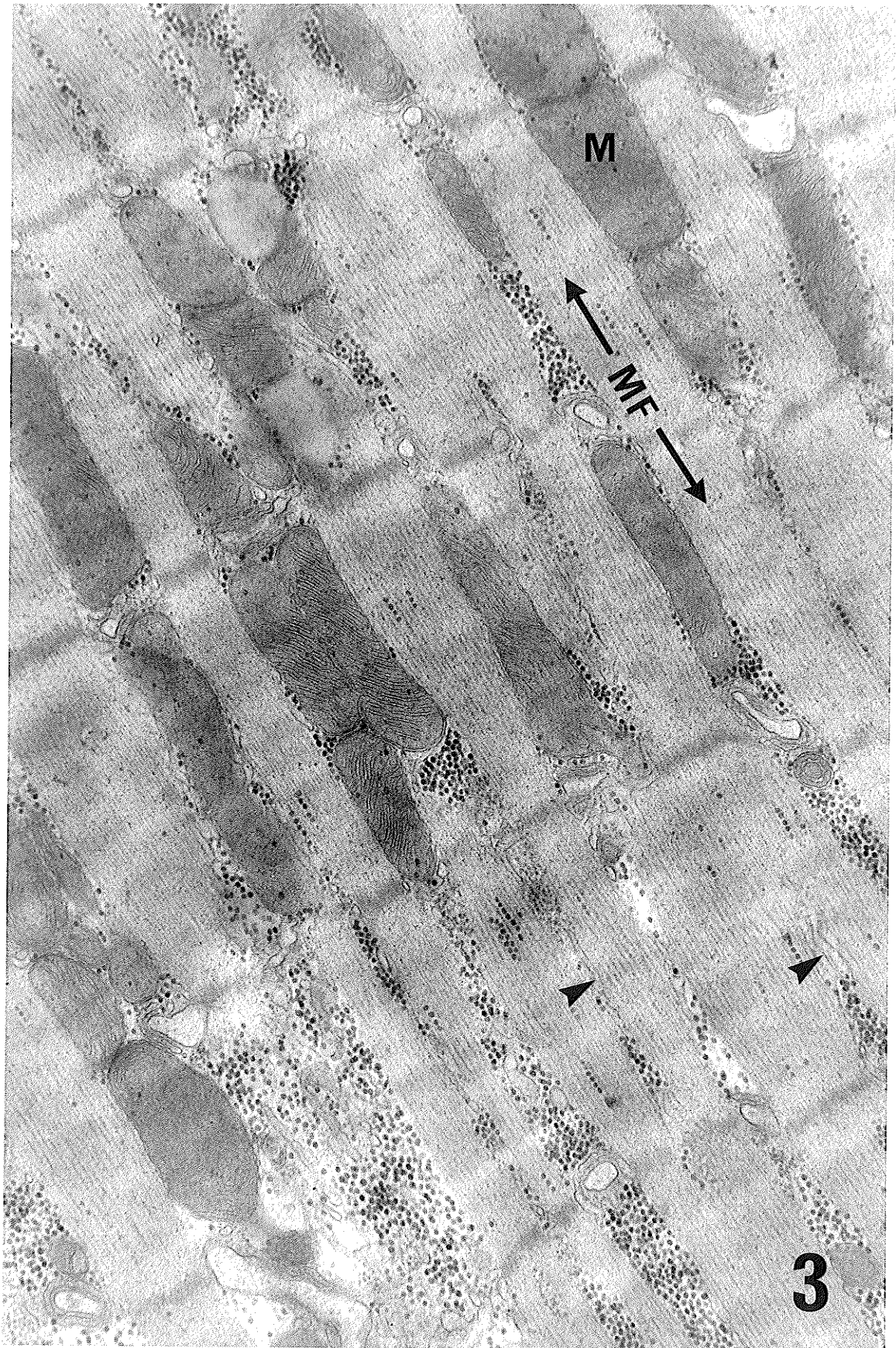
FIGURES 4 AND 5:

Myocardial cell damage in rats exposed to adriamycin:

Fig. 4: Shows swelling of mitochondria (m) as well as sarcoplasmic reticulum (arrow). Vacuolization (*) and lysosomal bodies (double arrow) are also apparent. X 15,000.

Fig. 5: Swelling as well as loss of cristae from the mitochondria. Some membrane cisternae in the dense bodies are seen. X 25,000.
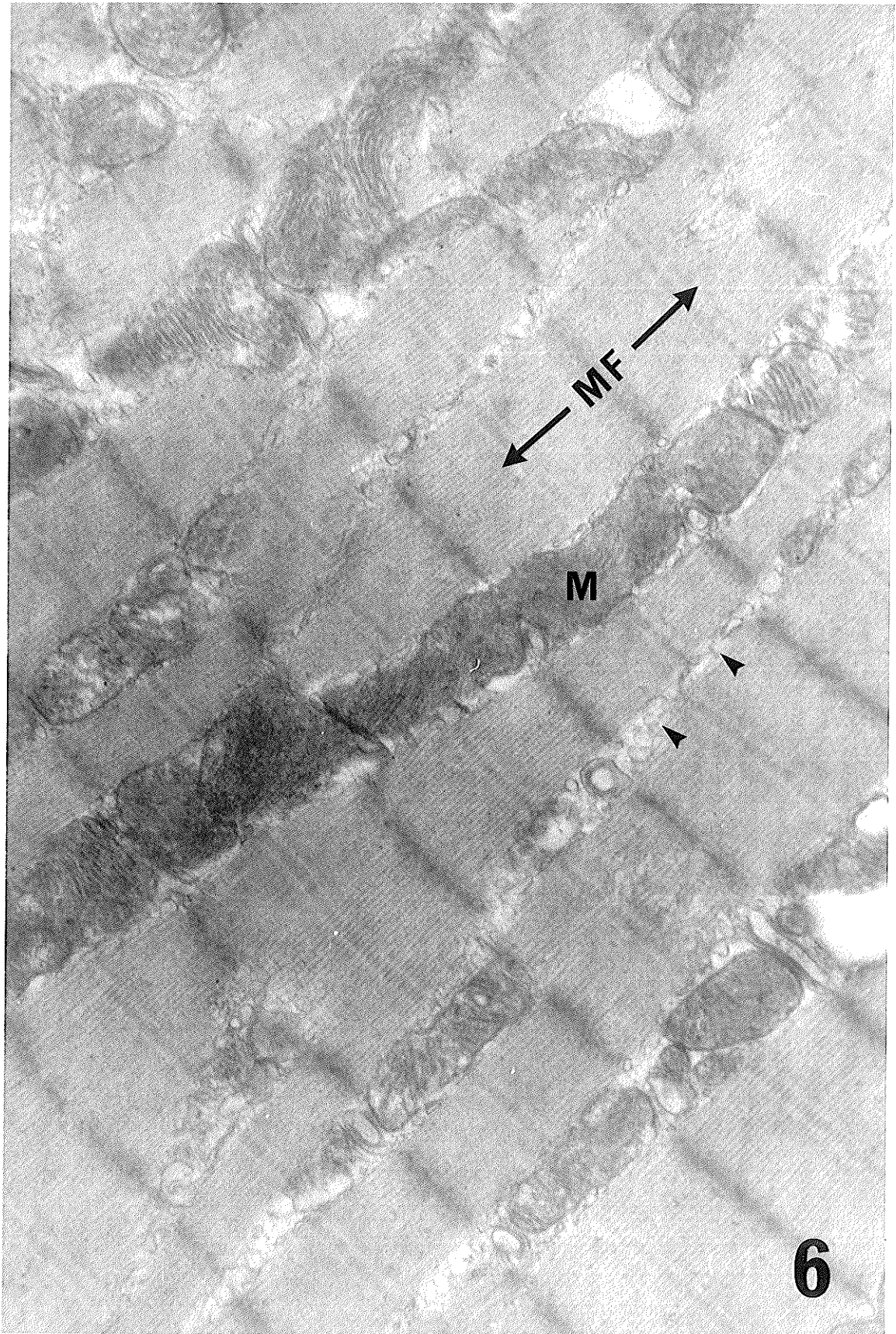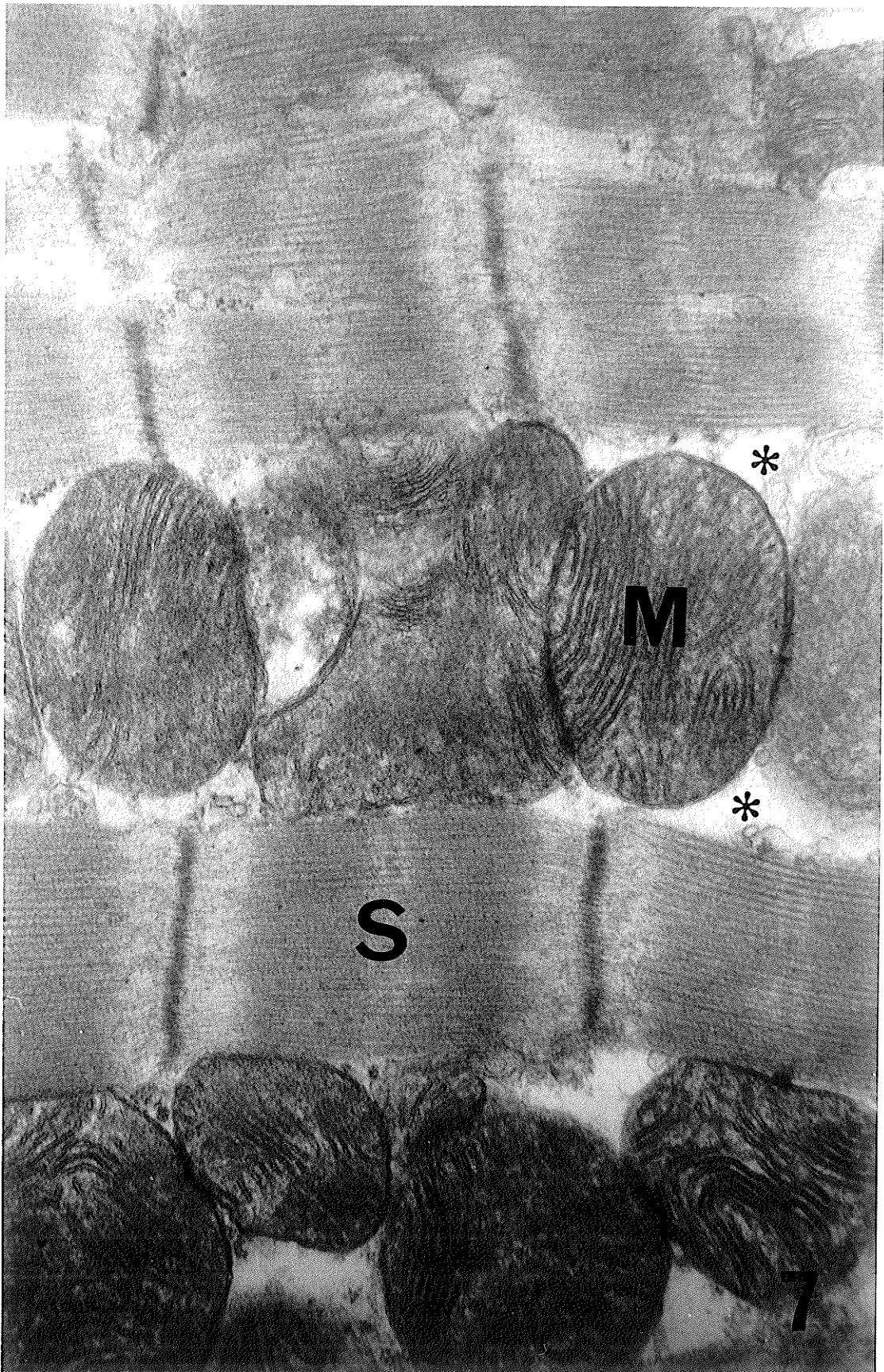
5

FIGURES 6 AND 7:

Portion of a myocardial cell from concurrently treated probucol and adriamycin treated rat:

Fig. 6:   Mitochondria (M), Myofibrils (MF), sarcoplasmic reticulum (arrows) and other cellular details are better maintained. X 12,000.

Fig. 7:   At higher magnification mitochondrial (M) cristae arrangement and sarcomeres (s) are quite normal. However, some perimitochondrial edema (*) is still apparent. X 25,000.

**FIGURES 8 AND 9:**

Portion of a myocardial cell from animals pretreated with probucol followed by concurrent treatment with probucol and adriamycin:

Fig. 8: Mitochondria (M); myofibrils, sarcoplasmic reticulum (arrows), completely maintained. X 15,000.

Fig. 9: Mitochondria and sarcomeres are normal. There is no edema in the perimitochondrial region as was seen in Fig. 7. X 25,000.

8

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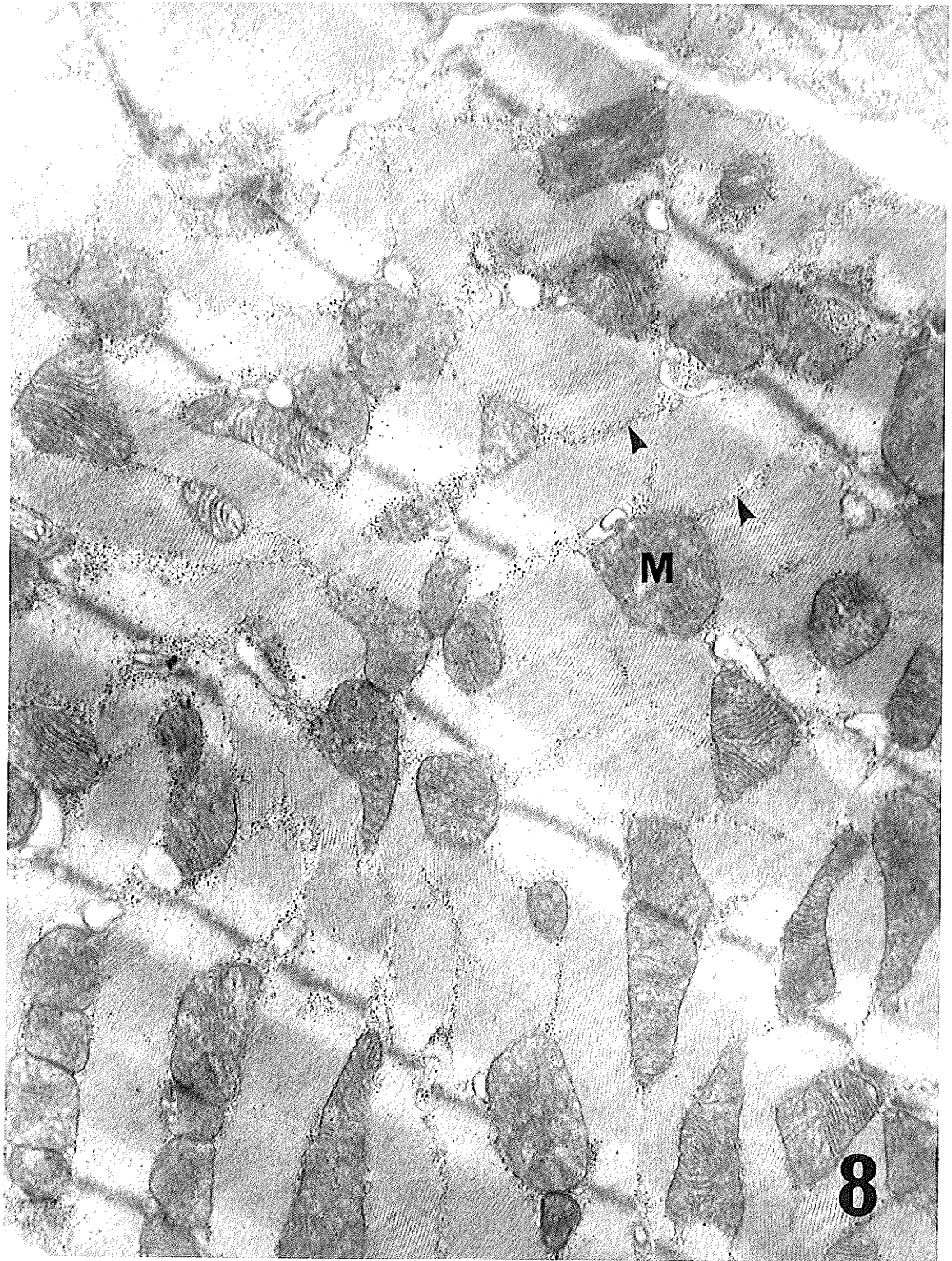
F. ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION

Different endogenous antioxidant enzyme activities were examined in the hearts from all groups in both parts of the study and these data are shown in Tables 5 and 6. Adriamycin treatment caused significant decrease (32%) in GSHPx activity compared to CONT group, while SOD and Catalase activities were not changed. Probucol treatment itself caused a significant increase in SOD activity with no change in the GSHPx or catalase activities. In PROB + ADR group, the GSHPx activity was near control levels while SOD activity showed a significant increase which was even more than the PROB group. Catalase activity was unaltered in the PROB + ADR group.

The amount of lipid peroxidation was determined by evaluating myocardial malondialdehyde (MDA) content and these data are also shown in Table 5. MDA levels in the ADR group were approximately 80% higher than the CONT group. In PROB and PROB + ADR groups, the MDA levels were not statistically different from CONT group.

In the second part of the study, with pretreatment as well as concomitant treatment with probucol, the only difference from the data shown in Table 5 was with respect to the PROB + ADR group (Table 6). In that group, GSHPx activity was about 20% higher than in CONT and PROB groups. Catalase activity did not show any change in any group. Total SOD activity in PROB and PROB + ADR group was significantly higher as compared to CONT and ADR groups. SOD values in the PROB group were higher by 40% than in the CONT and ADR group, while in the

61

PROB + ADR group SOD values were 70% higher than in the CONT and ADR group (Table 6).

MDA levels in CONT, PROB and PROB + ADR groups were not different from each other, while in the ADR group, MDA content was more than double compared to the CONT group.

TABLE 5: EFFECTS OF PROBUCOL (CONCURRENT) TREATMENT ON ADRIAMYCIN-INDUCED CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES AND LIPID PEROXIDATION.

Animal Group	GSHPx (nmol/mg prot)	SOD (U/mg prot)	Catalase (U/mg prot)	MDA (nmol/g heart)
CONT	59.9 ± 5.7	34.7 ± 4.4	31.3 ± 3.8	49.1 ± 3.2
ADR	40.7 ± 5.1*	41.0 ± 2.7	32.7 ± 2.0	82.1 ± 3.1*
PROB	52.4 ± 2.9	46.2 ± 6.5†	36.7 ± 3.2	54.3 ± 3.2
PROB + ADR	54.6 ± 5.3	64.1 ± 4.2†	30.0 ± 2.4	58.2 ± 7.1

Data are mean ± S.E. from 6-8 experiments. *) $P < 0.05$ different from all other groups. †) $P < 0.05$, different from CONT and ADR groups. CON, control; ADR, adriamycin; PROB, probucol; PROB + ADR, probucol and adriamycin; GSHPx, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde.

63

TABLE 6: EFFECTS OF PROBUCOL (PRE & CONCURRENT) TREATMENT ON ADRIAMYCIN-INDUCED CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES AND LIPID PEROXIDATION.

Animal Group	GSHPx (nmol/mg prot)	SOD (U/mg prot)	Catalase (U/mg prot)	MDA (nmol/g heart)
CONT	52.17 ± 2.5	34.52 ± 2.2	29.62 ± 3.6	48.10 ± 3.2
ADR	38.04 ± 3.2*	36.1 ± 4.3	33.33 ± 2.6	98.62 ± 4.5*
PROB	54.12 ± 2.3	48.65 ± 3.46†	31.12 ± 4.8	52.16 ± 4.1
PROB + ADR	65.15 ± 2.4†	58.16 ± 3.1†	30.06 ± 2.8	52.19 ± 2.3

Data are mean ± S.E. from 6-8 experiments. *) $P<0.05$ different from all other groups. †) $P<0.05$ different from CONT and ADR groups. CON, control; ADR, adriamycin; PROB, probucol; PROB + ADR, probucol and adriamycin; GSHPx, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde.

VI. DISCUSSION

Adriamycin (doxorubicin) is a broad spectrum anti-tumor antibiotic used to treat cancer patients. However, potential usefulness of this drug is currently limited by the development of a dose-dependent cardiomyopathic process terminating in severe heart failure (Lefrak et al., 1973; Minow et al., 1975; Singal et al., 1987; Praga et al., 1979). The cardiotoxic effects are manifested in three different forms: acute, subacute and chronic. In patients with adequate cardiac reserve, acute cardiac decompensation during adriamycin therapy is not commonly observed (Appelfeld and Egorin, 1984; Singal, 1985). Subacute effects including toxic myocarditis or pericarditis occur infrequently (Buja et al., 1973). Chronic changes develop in weeks to months of treatment and sometimes even after therapy has been completed. In order to avoid development of adriamycin-induced cardiomyopathy, the maximum cumulative dose of 550 mg/m^2 body surface area has been established. This dosage however, may not be sufficient in all patients as higher dosages are sometimes required for successful treatment of malignant diseases. Thus it carries the risk of deleterious cardiotoxic side effects. On the other hand, because of different risk factors, some patients may develop cardiomyopathy at the total cumulative dose of adriamycin well below 550 mg/m^2. Thus the use of this empirical dose has its limitations.

In reducing the risk of cardiomyopathy, one approach has been to synthesize adriamycin analogs that will have less or no cardiotoxicity and make the drug safer. So far, despite intense research, a truly better adriamycin analog has not been found

65

(Weiss, 1992). Another line of research has concentrated on finding substances which when co-administered with adriamycin will mitigate cardiotoxicity (Pristos et al., 1992; Von Hoff et al., 1981; Herman et al., 1981). Most promising findings were obtained with the drug ICRF-187. Because of a very serious bone marrow depression, this drug, however, can not be clinically used (Von Hoff et al., 1981). Thus the results observed so far, have been disappointing. The subchronic administration of adriamycin in the present study produced typical cardiomyopathy. Our study demonstrates for the first time that a simultaneous treatment with probucol mitigates adriamycin-induced cardiomyopathic changes as well as congestive heart failure as indicated by the improved cardiac structure, function and a reduced mortality in PROB + ADR group. The study suggests probucol to be a promising drug with a strong potential for combination therapy.

The structural changes in terms of loss of myofibrills, dilation of the sarcoplasmic reticulum and lysosomal changes seen in the present study have also been reported in the human biopsies (Lefrak et al., 1973; Bristow et al., 1978) as well as in other animals models (Jaenke, 1976; Singal et al., 1987). Adriamycin-cardiomyopathy is always accompanied by congestive heart failure which responds poorly to any therapeutical procedures and becomes progressively worse (Lefrak et al., 1973; Buja et al., 1973; Von Hoff et al., 1979). Cardiomyopathy seen in rats has also been reported to be refractory to positive inotropic treatments (Tong et al., 1991; Weinberg and Singal, 1986; Deally et al., 1990). Information obtained from the experimental model used in the present study, therefore, becomes even more

relevant from an applied viewpoint.

In the present study, in all adriamycin treated groups the most obvious effect was significant weight loss during the treatment period. Rats started gaining weight once the treatment period was over. These changes may relate to the transient loss of appetite due to the antibiotic effect. In fact, animal food consumption has been reported to be depressed during adriamycin treatment (Weinberg and Singal, 1986; Deally and Singal, 1990). In the adriamycin treated group, animals also developed large bellies and high quantities of ascites as a sign of congestive heart failure, which may also have contributed in the increase of their body weight during the post-treatment period. Inspite of a significant loss in body weight, there was still a drop in heart to body weight ratio which was explained by a significant loss in the heart weight itself.

A significant decrease in the aortic systolic (ASP) and left ventricular peak systolic (LVPSP) pressures as well as a significant increase in left ventricular end diastolic pressure (LVEDP) suggested depressed cardiac function. These data as well as ascites and congestive liver confirm the existence of the congestive heart failure which could not be compensated any longer with cardiac and/or extracardiac adjustments. Similar findings in rats exposed to adriamycin have been reported before (Weinberg and Singal, 1986; Deally et al., 1991). These animals were in a typical refractory congestive heart failure spiral (Singal et al., 1992). Increase in sympathetic tone in response to ADR stress has been reported in early stages of failure which may sustain systolic function at this stage. Ultimately this compensation

67

also becomes inadequate in late stages of failure (Tong et al., 1989). Ultrastructural damage is also extensive and may by itself explain refractoriness of the heart to therapeutical procedures. Simply, there is nothing left in the myocardium to respond to the positive inotropic agents or mechanical devices. In any case, depressed cardiac function as well as reduced responsiveness are suggested to be myogenic in origin (Deally et al., 1991). Probucol treatment had beneficial effects on the observed cardiac performance in that almost all measured parameters had improved back to the control levels. The only abnormal finding, seen in the concomitant probucol and adriamycin treatment group, was increased LVEDP.

Many studies have been devoted to understand the mechanism of these myocardial defects and thus of adriamycin cardiomyopathy. The efforts have resulted in several postulates including interaction of adriamycin with deoxyribonucleic acid and inhibition of nucleic acid as well as protein synthesis (Arena et al., 1974; Buja et al., 1973), release of vasoactive amines (Bristow et al., 1980), changes in adrenergic mechanisms (Tong et al., 1991), abnormalities in high energy phosphate metabolism (Gosalvez et al., 1979), free radical formation and lipid peroxidation (Doroshow, 1983; Kalyanaraman et al., 1980; Singal et al., 1987; Singal et al., 1985), alterations in sarcolemma and membrane bound enzymes (Singal and Pierce, 1986; Singal et al., 1984; Singal and Panagia, 1984), lysosomal alterations and imbalance of myocardial electrolytes (Singal et al., 1985; Olson et al., 1974) as well as occurrence of calcium overload (Singal and Panagia, 1984). Although a number of different factors may play a role in the pathogenesis of this disease, increased free

radical stress due to adriamycin treatment appears to be a common factor (Singal et al., 1987; Hasinoff et al., 1988; Costa et al., 1988; Doroshow , 1983; Kalyanaraman et al., 1980). Because of the semiquinone moiety in the adriamycin molecule, it is reported to increase the oxygen radical activity and peroxidation of polyunsaturated fatty acids that may explain adriamycin-induced defects in membrane function due to this drug. In this regard, probucol is known to have antioxidant properties which may have contributed to the protective effect (Mao et al., 1991; Pryor et al., 1988).

Probucol in the plasma is transported predominantly by LDL, VLDL and HDL (Zimetbaum et al., 1990). Oral administration of probucol at a dose of 1 gm/day, increases its level in the blood as well as adipose tissue (Taylor et al., 1978). However, there seems to be no absolute correlation between the plasma levels of probucol and the extent of cholesterol lowering effect (Polachek et al., 1970). Although it is difficult to draw any parallel between the dosage used by us in rats (6 x 10 mg/kg, i.p.) and therapeutic dosage (2 x 500 mg/day, 3-6 months), the probucol treatment protocol used in our study was well tolerated.

Probucol treatment in heterozygous familial hypercholesterolamia caused the regression of xanthomas which did not correlate with the level of cholesterol reduction (Yamamoto et al., 1986). Cholestyramine, another cholesterol lowering drug, and probucol, both sharply lowered the serum cholesterol levels in nonhuman primates while only probucol caused regression of atherosclerotic lesions (Wissler and Vesselinovitch, 1983). These observations clearly suggest that beneficial effects of probucol may be independent of cholesterol lowering effect. Because of the two

phenolic groups in its molecular structure, probucol has been reported to be a strong antioxidant (Mao et al., 1991; Pryor et al., 1988) and it appears that the protection offered by probucol in this study may involve antioxidant mechanisms. In this regard, adriamycin has been shown to promote the production of free radicals (Doroshow, 1983; Kalyanaraman et al., 1980) and these toxic species are known to cause myocardial dysfunction (Gupta and Singal, 1989). Data on lipid peroxidation is also in concert with this suggestion inasmuch as probucol caused a significant attenuation in the ADR-induced increase in MDA levels. Beneficial effect of probucol against restenosis after percutaneous transluminal coronary angioplasty has also been suggested to be due to its antioxidant property (Schneider et al., 1993).

Development of adriamycin cardiomyopathy suggested an increase in the free radical production and/or decrease in endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx). In our study, ADR group had significant decrease in GSHPx. Cardiac GSPHx was also reported to be inhibited (Doroshow et al., 1980) as well as depleted by adriamycin (Olsen et al., 1980). In our study, probucol prevented adriamycin-induced decrease in GSHPx activity.

Probucol by itself caused an increase in SOD level and the observed increase was even higher (88%) in probucol + adriamycin treated group. Thus, probucol clearly improved "endogenous antioxidant reserve", and the latter has been suggested to improve myocardial structure and function (Singal and Kirshenbaum, 1990). It is important to note that there was a small, though statistically not significant, increase

in the SOD activity in the ADR group. Induction of SOD activity by adriamycin in lymphocytes and neutrophils was described by other authors (Niwa et al., 1993). Hence, this high increase in SOD activity in PROB + ADR group may be due to some synergistic effect between adriamycin and probucol. Catalase activity did not show any change in any of the four groups and this activity is generally low in the heart (Doroshow et al., 1980). Mechanisms for adriamycin-induced decrease in GSHPx and probucol-induced increase in antioxidants (GSHPx and SOD) are not clear. Present study, however, clearly demonstrates that probucol may be providing some protection by acting as an antioxidant as well as by promoting endogenous antioxidants. In this regard, the LDL isolated from the plasma of patients treated with probucol was resistant to oxidative modification (Parthasarathy et al., 1986), supporting the increased antioxidant ability in our experimental animals.

Peroxidation of membrane lipids by different drugs has been reported in many different pathological situations (Plaa and Witsche, 1976). Because of peroxidation of polyunsaturated fatty acids, changes in membrane microarchitecture and permeability as well as alterations in membrane-bound enzyme activities occur (Kaul et al., 1993). Adriamycin has a high affinity for cardiolipin, a substance found in large amounts in mitochondrial membranes. Hence, mitochondria are particularly susceptible to adriamycin-induced peroxidation. Observed decrease in MDA levels in PROB + ADR treated groups lends support to this concept. Structural integrity, including that of mitochondria, was maintained by probucol. Hearts from the PROB + ADR group showed regular myofibrilar structure, maintained sarcotubular

71

reticulum and preserved mitochondria. Although in concomitant with PROB + ADR treatment group, tremendous improvement in myocardial ultrastructure was seen, some intracellular edema was still noticeable around the mitochondria at higher magnification. Presence of residual structural injury as well as higher than minimal LVEDP suggested only partial protection. However, pretreatment with probucol prior to concomitant adriamycin and probucol treatment helped further in the prevention of development of adriamycin-induced cardiomyopathy. In probucol pretreatment group, no mortality, normalisation of LVEDP to control levels, and ultrastructure indistinguishable from control animals were seen.

In conclusion, this study demonstrates that ADR cardiomyopathy is associated with an antioxidant deficit and that treatment with probucol offers protection against adverse side effects of adriamycin. Concurrent treatment with probucol significantly ameliorated the development of cardiomyopathy. However, a better protection was seen with pre as well as concurrent treatment. Improved cardiac function due to treatment with probucol may be related to the maintenance of the antioxidant status of the heart. Though the precise mechanism by which probucol provides protection is not known, our data have indicated that probucol not only augments the endogenous antioxidants but may also act as an antioxidant by itself. Further studies will be needed to elucidate the mechanisms by which probucol influences endogenous antioxidant activities. For a possible clinical use of probucol-adriamycin combination in the treatment of cancer patients, effects of probucol on adriamycin anti-tumorigenicity need to be defined. Hopefully studies in the future will address this issue.

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81

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88

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