sacs with small atrophic ovaries; and hilus cell tumors of the ovary. Control animals that received oil injections did not manifest any reproductive tract abnormalities and were cyclic. The types and frequency of abnormalities varied widely among the various treatment groups. The high dose of either Clomid (500 μg) or Nafoxidine (100 μg) produced some form of abnormality in 80 to 100 percent of the animals. Although intermediate and lower doses have not been completely evaluated, our results indicate that 10 to 50 percent of the animals will be adversely affected. The tumor that is shown in Fig. 1 was found in an animal that was injected with only 2 μg of Nafoxidine. Uterine metaplasia and infertility accompanied by polycystic degeneration of the ovary have been described by others (*5*).

The variation in the kinds of abnormalities probably relates to both the dose of the compound and the age of the animal to which it is administered. The ability of the reproductive organs to respond to estrogenic compunds depends, in part, on the presence of estrogen receptors, and the concentration of these receptors is known to increase with time in the neonatal rat (*6*). Therefore, the effectiveness of the compound, as well as its mode of action, may vary with time.

Although we have not ruled out the possibility of indirect effects of Nafoxidine and Clomid, it seems likely that these drugs are acting directly on the various target tissues. We have observed previously that Nafoxidine causes long-term retention of the estrogen receptor by uterine nuclei in immature rats (21 to 23 days old). This long-term retention is accompanied by a sustained stimulation of uterine growth, up to 19 days after a single injection (*3*). Our data indicate that this effect is also operating in the neonatal rat; therefore, we think that the abnormalities that we have observed are due to a similar long-term estrogenic stimulation. Whether such nuclear binding and estrogenic stimulation occurs in adult animals or in other species remains to be resolved.

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3. Clomid and Nafoxidine have traditionally been considered to be estrogen antagonists; however, we have shown that they can function as long-acting estrogens in the immature rat uterus. [J. H. Clark, J. N. Anderson, E. J. Peck, Jr., *Steroids* **22**, 707 (1973)]; J. H. Clark, E. J. Peck, Jr., J. N. Anderson, *Nature (London)* **251**, 446 (1974); J. H. Clark, Z. Paszko, E. J. Peck, Jr., *Endocrinology* **100**, 91 (1977).
4. Each compound was dissolved in absolute ethanol, stirred into warmed (about 45°C) sesame oil until the ethanol had evaporated, and injected subcutaneously in the nape of the neck. Clomid is a mixture of *cis* and *trans* isomers and was used as such because this is the form which is administered to women.
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7. Supported by the American Cancer Society grant BC-92. We thank L. Swaine for assistance with the photography and G. Brady for technical assistance.

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## Adriamycin: The Role of Lipid Peroxidation in Cardiac Toxicity and Tumor Response

Abstract. *The antitumor antibiotic, adriamycin, induces severe cardiac toxicity associated with peroxidation of cardiac lipids in mice. Both this lipid peroxidation and cardiac toxicity of adriamycin are reduced by prior treatment of the animals with the free radical scavenger tocopherol. Such treatment with tocopherol does not, however, alter the magnitude or duration of the adriamycin-induced suppression of DNA synthesis in P388 ascites tumor, nor does it diminish the antitumor responsiveness of P388 ascites tumor. These results suggest that adriamycin has at least two mechanisms of tissue damage: one, which involves lipid peroxidation, is blocked by tocopherol and results in cardiac toxicity; the other, which involves binding to DNA, is not antagonized by tocopherol and is responsible for tumor response.*

The antitumor antibiotic, adriamycin, is one of the most important new drugs in the field of cancer chemotherapy; it has exhibited activity against a wide spectrum of human neoplasms and in particular against solid tumors. Unfortunately, its clinical use has been compromised by an unusual and potentially lethal cardiac toxicity, which is thus far unexplained (*1*).

Handa and Sato (*2*) have recently demonstrated in microsomes exposed to anthracyclines a superoxide radical ion production that is dependent on reduced nicotinamide adenine dinucleotide phosphate. Previous investigators have shown that superoxide radical ions can decompose to yield hydroxyl radicals, peroxy radicals, and hydrogen peroxide (*3*). These, in turn, are known to initiate free radical mediated chain reactions which result in conversion of the membrane unsaturated fatty acids to lipid peroxides (*4*). We have recently reported that in mice treated with tocopherol, an effective free radical scavenger known to inhibit the formation of lipid peroxides, the toxicity of adriamycin is significantly reduced (*5*), a lethal dose for 85 percent of the animals being converted to a lethal dose for 10 percent. These results suggested that lipid peroxidation may play an important role in adriamycin toxicity.

In the present study, experiments

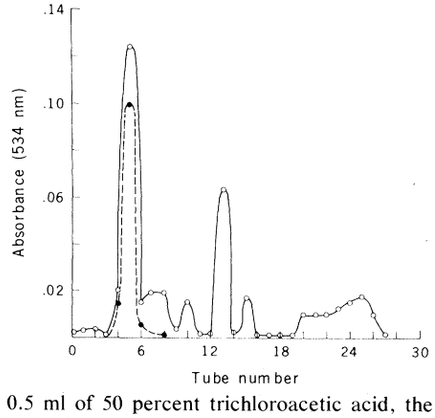


Fig. 1. Detection of malondialdehyde in murine cardiac tissue after the administration of adriamycin. Male mice ($CDF_1$; 20 to 25 g) were housed in a constant temperature environment, caged on hardwood bedding and fed Wayne F6 Lab-Blox. Each mouse (three groups of three mice each) received an intraperitoneal injection of adriamycin (15 mg/kg); 4 days later the animals were killed by cervical dislocation. The hearts were rapidly removed, washed in iced phosphate-buffered saline, *p*H 7.4, blotted dry, and placed in a weighed vial. After weighing, each group of three hearts was disrupted with a Polytron homogenizer in 2 ml of 0.02*M* potassium phosphate buffer, *p*H 7.4, containing butylated hydroxytoluene (0.5 mg/100 ml) to prevent further oxidation of lipids. After the addition of 0.5 ml of 50 percent trichloroacetic acid, the samples were heated to 90°C for 15 minutes. The samples were reduced to 0.5 ml by lyophilization, brought to *p*H 7.5, applied to a Sephadex G-10 column (0.9 by 40 cm), and eluted with 0.05*M* tris-HCP buffer, *p*H 7.4, containing 0.1*M* NaCl; 1-ml fractions were collected. After 300 μl of 1 percent 2-thiobarbituric acid (TBA) was added to 300 μl of each fraction, the mixture was heated to 90°C for 15 minutes and absorbance was read at 533 nm. The TBA-reactive material from tissue extracts (○) is compared with a malondialdehyde standard synthesized from tetraethoxypropane (K and K Chemical) (●) (*6*). The concentration of malondialdehyde was then calculated with a molar extinction coefficient of $1.5 \times 10^5$ being used.

were directed at detecting the formation of lipid peroxides in adriamycin-treated animals. These peroxides are formed from oxidation of naturally occurring unsaturated fatty acids and rapidly decompose in vivo to yield a variety of products, the best characterized of which is malondialdehyde (*6*). Using the procedure outlined in Fig. 1, we were unable to detect malondialdehyde in acid extracts of normal murine cardiac tissue. However, malondialdehyde was readily detected 2 days after the intraperitoneal injection of adriamycin (15 mg/kg), and the compound reached a peak concentration of 53 ± 10 $\mu M$ by 4 days (Fig. 1) before disappearing by day 6. This wave of malondialdehyde formation did not occur in the cardiac tissue of animals treated with an intraperitoneal injection of tocopherol (85 units; 920 units per gram; Sigma) 24 hours prior to the injection of adriamycin.

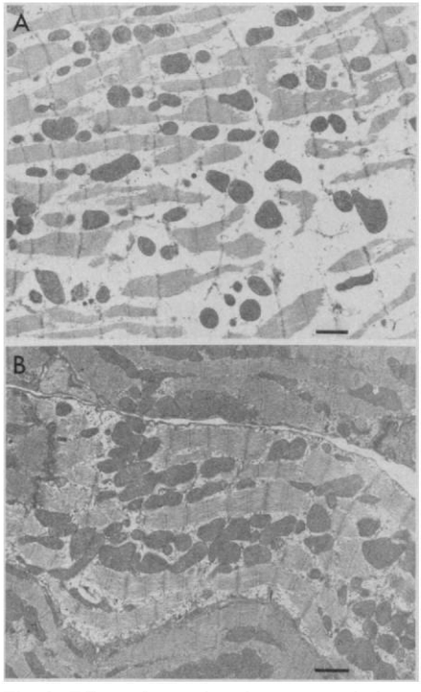


Fig. 2. Effect of tocopherol on cytopathology of adriamycin-induced cardiomyopathy. Three groups of three male mice ($CDF_1$; 20 to 24 g) were used; group 1 was used as a control; group 2 received 85 units of tocopherol intraperitoneally 24 hours prior to receiving an intraperitoneal injection of adriamycin (15 mg/kg); and group 3 received adriamycin (15 mg/kg) alone. The animals were killed on day 5 by cervical dislocation. The left ventricles were removed, minced into 1-mm samples, and fixed at room temperature for 5 hours in 2 percent paraformaldehyde, 2.5 percent glutaraldehyde in cacodylate buffer, *p*H 7.4. The samples were again fixed in 2 percent osmium tetroxide for 2 hours, dehydrated in graded ethanol, and embedded in Epon 812. Sections (60 to 100 $\mu$m) were cut in a Porter-Blum MT-2B ultramicrotome, double-stained with uranyl acetate and lead citrate, and examined in a Philips EM201 electron microscope. (A) Section showing the changes in group 3, which received adriamycin only. (B) Section showing a focal lesion surrounded by normal heart tissue from group 2, which received both tocopherol and adriamycin (scale bars, 1 $\mu$m).

By means of electron microscopy we obtained histological evidence that adriamycin-induced cardiomyopathy is prevented by tocopherol. Figure 2 shows that the magnitude and incidence of myofibrillar fragmentation, mitochondrial disruption, glycogen depletion, and edema, usually seen in cardiac tissue from anthracycline-treated mice (*7*), were reduced in animals that were first treated with tocopherol. Each of these four types of changes was scored in coded electron photomicrographs against a scale of 0 to 4, in which the highest score indicated the most severe change. The sum of the scores for each sample was taken to be an index of the overall severity of the cardiomyopathy. Mean scores (± standard error) of 13.8 ± 3.0 for the group given adriamycin only and 2.4 ± 0.9 for the group receiving both adriamycin and tocopherol ($P < .05$) demonstrated that prior treatment with tocopherol significantly reduced the adriamycin-induced cardiomyopathy, probably by blocking lipid peroxidation.

Adriamycin binds to mammalian DNA (*8*), causing fragmentation of DNA and inhibition of DNA synthesis. This DNA binding has been proposed as a mechanism for the antitumor effect of adriamycin (*9*). For this reason, we have tested the impact of tocopherol on the suppression of DNA synthesis in tumors after adriamycin administration. The P388 ascites tumor was used in these studies because it is the most sensitive to the standard transplantable murine tumors to adriamycin (*10*). From Fig. 3, it is apparent that prior treatment of the animals with tocopherol does not diminish the ability of adriamycin to inhibit DNA synthesis in P388 ascites tumor tissue.

In addition, using the techniques outlined in Fig. 1, we have been unable to detect malondialdehyde formation in the P388 ascites tumor over a 6-day period after the administration of adriamycin as in Fig. 3. To the extent that DNA binding rather than lipid peroxidation was the important determinant of tumor response, prior treatment with tocopherol would also not be expected to influence the sensitivity of that tumor to adriamycin.

To test this, we studied the effect of tocopherol administration on the responsiveness in vivo of the P388 ascites tumor to adriamycin. In this system, mice that are not treated with tocopherol survive 7.4 ± 0.3 days (± standard error) after the transplantation of $10^6$ tumor cells. Tocopherol alone had no effect on survival of these animals. In mice treated first with tocopherol, survival after a 7.5 mg/kg dose of adriamycin was 18.3 ± 0.7 days, nearly identical to the comparable group treated with adriamycin alone which survived 18.4 ± 0.6 days (*11*). At doses of 10, 12.5, and 15 mg of adriamycin per kilogram, the mice with and without tocopherol treatment survived 21 ± 1 days versus 13.8 ± 0.9 days, 17.8 ± 0.8 days versus 13.1 ± 0.5 days, and 12.8 ± 0.6 days versus 8.0 ± 0.4 days, respectively. In each case, the group treated with tocopherol survived significantly longer ($P < .01$) than the

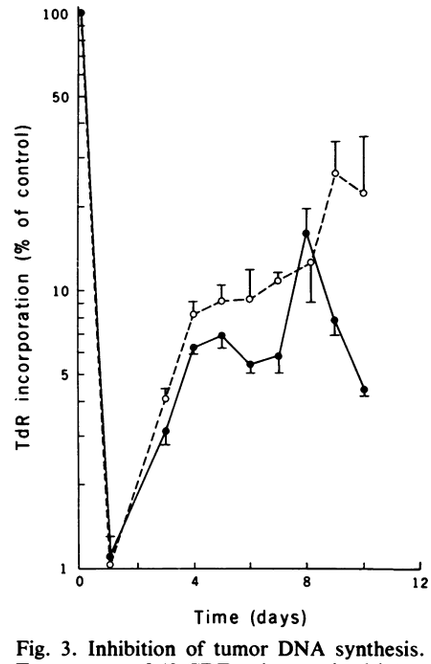


Fig. 3. Inhibition of tumor DNA synthesis. Two groups of 40 $CDF_1$ mice received intraperitoneal injections of adriamycin (10 mg/kg); one group was previously treated with 85 units of tocopherol, the other received only the adriamycin. At each of the time intervals indicated, four mice received intraperitoneal injections of 100 $\mu$c of tritiated thymidine (TdR). One hour later, the mice were killed, the ascitic tumor cells were obtained by lavage of the peritoneum with iced phosphate-buffered saline (0.85 percent NaCl, *p*H 7.4). After centrifugation at 700*g* for 15 minutes, the DNA was extracted and its specific activity [in disintegrations per minute (dpm) per milligram of DNA] was measured according to the techniques described (*7*). The results for the groups receiving adriamycin and tocopherol (•) or adriamycin alone (○) were expressed, respectively, as a percentage (± standard error) of the incorporation of label into the DNA of animals that had been treated or not treated previously with tocopherol and had not received adriamycin. The mean incorporation of tritiated thymidine for the control animals was 23,340 dpm per microgram of DNA, and that of animals treated with tocopherol alone was 21,134 dpm per microgram of DNA.

comparable group given adriamycin only. These results indicate that treatment with tocopherol does not impair the responsiveness of the P388 ascites tumor to adriamycin. Further, because it diminishes the dose-limiting toxicity of adriamycin, larger doses of the drug may be administered, leading to a significantly improved therapeutic index. These results provide further evidence in favor of the hypothesis that adriamycin may have at least two mechanisms of tissue damage in the mouse. One, which involves lipid peroxidation, is blocked by tocopherol and appears to play a major role in the development of cardiomyopathy. The other, which may involve binding of adriamycin to DNA, is unaffected by tocopherol and appears to be the major determinant of cytotoxicity for P388 cells. Additional work is needed to determine if any human tumors parallel P388 in these respects. To the extent that a similar differential exists between human cardiac tissue and human tumor, pretreatment with tocopherol or other free radical scavengers might be of therapeutic benefit.

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11 February 1977

## Experimental Infarct Sizing Using Computer Processing and a Three-Dimensional Model

Abstract. *A method for noninvasive sizing of myocardial infarction, in which data from technetium-99m stannous pyrophosphate scintigrams and a three-dimensional model were used, was tested on experimental, acute anterior infarcts in dogs. The results indicate that the method does size experimental anterior infarcts accurately, but further testing will be necessary to assess the capabilities of the technique for sizing other types of infarcts.*

The major cause of death in patients reaching the hospital with acute myocardial infarction is "pump failure" that occurs as a complication of a large myocardial infarct and is often expressed clinically as cardiogenic shock, medically refractory congestive heart failure, or medically refractory ventricular arrhythmias, or a combination of all (*1*). Hence the ability to size acute myocardial infarcts appears to be important. Quantitative infarct measurements could potentially be used to help assess patient prognosis and to aid in planning subsequent treatment. Myocradial scintigrams, with technetium-99m stannous pyrophosphate ($^{99m}$Tc-PYP) as an imaging agent, are used to identify and localize acute myocardial infarcts in both experimental animals and in patients (*2*). Since injured myocardial tissue is identified as a bright spot on a $^{99m}$Tc-PYP scintigram, information obtained from such scintigrams could probably be used to size infarcts in patients, provided a suitable sizing technique could be developed.

The $^{99m}$Tc-PYP myocardial imaging has been used to estimate the size of acute anterior infarcts in experimental animals, but previous estimates have employed "areas of increased activity" from one scintigraphic view as a measure of infarct size (*3*). Area seems to be a relatively poor measure, since it is a two-dimensional quantity and does not take into account the three-dimensional nature of the infarcted tissue. In particular, since infarcts occur in various positions and orientations, no simple two-dimensional measurement will be able to size all types of infarcts successfully. Therefore, we have attempted to develop a relatively simple method for reconstructing infarct size in three dimensions, using the noninvasive $^{99m}$Tc-PYP myocardial scintigram.

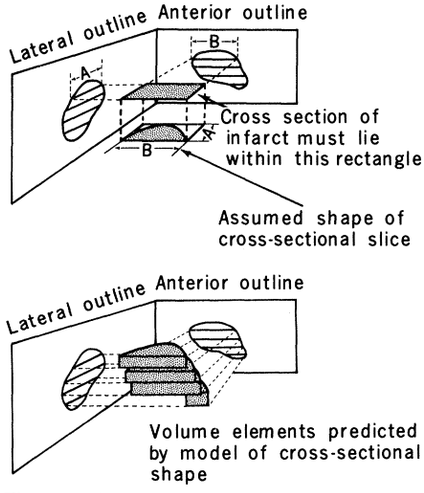


Fig. 1. The model we utilized (C-shaped cross-sectional model) and actual data obtained from two orthogonal projections of infarct outlines. See text for details.

Several methods for reconstructing approximate cross sections of three-dimensional objects from their projections have been described (*4*). Reconstruction of myocardial infarcts from $^{99m}$Tc-PYP images is a formidable task, however, for two reasons. (i) The $^{99m}$Tc-PYP uptake is not uniform in infarcted tissue (*2*), and therefore the intensity levels in scintigrams do not accurately indicate the extent of infarction, merely its presence. (ii) Only three or four scintigraphic projections are obtained during routine examination; hence not enough scintigraphic views are available to permit sizing by use of such reconstruction algorithms as those utilized in computerized transaxial tomography (*4*).

Since we do not have enough projections to reconstruct the actual infarct shape, our method uses a three-dimensional "model" of the infarct, and, since intensity levels in the scintigrams can be misleading, we have fit the model according to the infarct boundaries obtained from the scintigram views. Defining infarct boundaries in a $^{99m}$Tc-PYP scintigram can also be a difficult problem because the infarct may be partially obscured by the presence of overlapping bones in the images. Computer preprocessing may be useful when isolating an infarct in a scintigraphic image in order to filter the bones from the image and to improve contrast (*5*). However, the preprocessing step must preserve infarct boundaries as much as possible.

The infarct-sizing method we have developed makes use of a slight modification of an elliptical-slice method that has already been described (*6*). Our modifi-