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In Vivo RNA Synthesis in the Hearts of Adriamycin-treated Rats[1]

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ABSTRACT

Data are presented which demonstrate the effect of both acute and chronic Adriamycin treatment on myocardial RNA synthesis *in vivo*. The results suggest that the inhibition of RNA synthesis is a transient effect following both types of treatment and that the degree and duration of this effect is dose dependent. The data further demonstrate that Adriamycin does not alter the turnover rate of RNA and that both messenger RNA and ribosomal RNA are affected. These results, interpreted in conjuction with pharmacokinetic data, permit speculation on the relationship between acute and chronic Adriamycin treatment as well as cellular ramifications with regard to the protracted, dose-dependent cardiomyopathy.

INTRODUCTION

ADR[3] and several other anthracycline antibiotics used in the treatment of various cancers exhibit a variety of toxic side effects involving the heart, kidney, and lymphohemopoietic tissues. Understanding of the biochemical mechanism for the ADR-induced cardiomyopathy is incomplete. The heart tissue of ADR-treated animals is observed to undergo a variety of biochemical changes which include an increase in tissue calcium level (14), inhibition of the mitochondrial respiratory enzyme CoQ_{10} (10), inhibition of sodium-potassium ATPase (7), and an increase of free radicals through lipid peroxidation (12). Tissue culture and *in vitro* experiments have demonstrated a myriad of other ADR-induced biochemical changes. Unfortunately, no single biochemical observation unequivocally explains the pathogenesis of the ADR-induced cardiomyopathy.

The mechanism of ADR action in actively replicating cells is generally thought to occur through inhibition of DNA synthesis by intercalation of drug into DNA (4, 16). However, since adult myocardial cells are mitotically static, DNA intercalation might be expected to primarily affect DNA-dependent RNA polymerase activity. It has indeed been demonstrated (17) that ADR inhibits RNA synthesis at concentrations comparable to that required to block DNA synthesis. Disturbances in RNA metabolism should have profound effects on the normal utilization of genetic information and this, in turn, would affect the functional state of cells.

The purpose of this study was to investigate the effects of both acute and chronic *in vivo* ADR administration on myocardial RNA synthesis to determine if alterations in this pathway might provide insight into the mechanism of the anthracycline antibiotic-induced cardiomyopathy.

MATERIALS AND METHODS

Animals and Drug Treatment. Male Sprague-Dawley rats initially weighing approximately 150 g were used in all experiments. ADR was injected i.v. at a dose and schedule depending on the type of experiment. For acute experiments, animals received 2 injections of 2.5 mg ADR per kg body weight, with the second injection administered after an 8-hr interval (double injection schedule). In subacute experiments using 3 multiple double-injection schedules requiring survival times of up to 144 hr posttreatment, ADR was administered at the rate of 1.25 mg/kg body weight. To study chronic drug effects, ADR was administered at a dose of 2.2 mg/kg body weight once a week for 6 weeks.

In order to utilize the same control data for each experiment when evaluating the effect of different ADR treatment doses or postinjection time intervals upon RNA synthesis, the starting time of each treatment group within an experiment was staggered in order to permit termination and sacrifice of all animals to occur simultaneously. Thus, all animals received [^{3}H]uridine (50 μCi/100 g body weight i.v.) at the same time and incorporated label during the same 17-hr labeling period.

Measurement of RNA Synthesis. RNA synthesis was measured by the amount of [5-^{3}H]uridine incorporated during a 17-hr interval. At the appropriate time following the final drug injection, each animal was given 50 μCi [^{3}H]uridine (New England Nuclear; 500 mCi/mmol; 2 mol/ml) per 100 g body weight. A control group of animals was similarly treated. Seventeen hr post-isotope injection, animals were killed by cervical dislocation. The heart was excised, and the ventricles and septum were isolated and coarsely minced. The minced tissue was thoroughly washed with 0.25 M sucrose in 0.05 M Tris-Cl (pH 7.5 at 20°)-0.25 M KCl-0.005 M $MgCl_2$ buffer.

The amount of RNA and DNA were measured spectrophotometrically as nucleotides using the procedure of Fleck and Munro (6). Coarsely minced tissue was suspended in 0.25 M sucrose in 0.05 M Tris-Cl (pH 7.5 at 20°)-0.25 M KCl-0.005 M $MgCl_2$ buffer at approximately 200 mg/ml. The tissue was homogenized at 0° with a Tekmar Tissumizer (Tekmar Co., Cincinnati, Ohio) using two 15-sec treatments (Setting 49 with a TR-5T high-torque speed controller). To 1.2 ml of the homogenate, 0.8 ml of 0.6 N PCA was added to precipitate DNA, RNA, and protein. Precipitated material was collected by centrifugation (6000 × *g*; 5 min at 4°), and the precipitate was washed twice with 0.2 N PCA. The combined supernatant fluids represented the acid-soluble pool. From the PCA-precipitated material, RNA was hydrolyzed by incubation for 1 hr at 37° in 4 ml 0.3 N NaOH. After the samples were cooled to 0°, DNA and protein were precipitated by adding 1.8 ml 2.4 N PCA. Precipitated material was collected by centrifugation, and the supernatant fluid, representing RNA nucleotides, was transferred to a clean tube for subsequent analysis. DNA was hydrolyzed from the precipitated material by incubation for 20 min at 90° in 4 ml 0.6 N PCA. After cooling, unhydrolyzed material was removed by centrifugation and discarded. The supernatant fluid represented DNA nucleotides. The concentration of DNA and RNA nucleotides was determined by spectroscopy at 260 nm.

Radioactivity Determination. Radioactivity in the acid-soluble pool was determined by counting a 0.5-ml sample in 10 ml Biofluor (New England Nuclear, Boston, Mass.). Similarly, RNA radioactivity was measured in a 1-ml sample dissolved in 15 ml Biofluor. All samples were counted in a Beckman LS-200 liquid scintillation counter with a ^{3}H counting efficiency of 51%.

RESULTS

Acute ADR Effects. The treatment schedule used in this

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[3] The abbreviations used are: ADR, Adriamycin; PCA, perchloric acid; poly(A)+, polyadenosine-containing; poly(A)−, polyadenosine-lacking; poly(A), polyadenosine.

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study to characterize the acute effect of ADR on myocardial RNA synthesis consisted of i.v. injections of ADR in 2 doses of 2.5 mg/kg each administered at 8-hr intervals. The selection of these particular conditions was based entirely on experimental pragmatism; *i.e.*, those conditions which produced the greatest inhibition of RNA synthesis with the least animal loss due to acute toxic overdose. As a first approximation, the inhibition of RNA synthesis as a function of the double-injection schedule is represented in Chart 1. This double reciprocal plot of the data demonstrates that the treatment schedule chosen represents about one-half the maximal inhibition (K_D 3.19).

In order to further characterize the inhibition of RNA synthesis after acute treatment, the incorporation of RNA precursor was measured at 3 time intervals after a single double injection of ADR (Table 1). In treated Group A, rats received labeled RNA precursor in conjunction with their second ADR administration, while Groups B and C received isotope 24 and 48 hr, respectively, after the second ADR injection. All rats, including controls, were sacrificed 17 hr after radiolabeling. ADR produced a significant inhibition of RNA synthesis in this experiment which could be detected up to 24 hr postinjection (Groups A and B). However, this inhibition appeared to be transient since labeling at 48 hr posttreatment resulted in no significant decrease in RNA synthesis rate. This response to [^{3}H]uridine incorporation was not attributable to changes in RNA precursor pool specific activity.

Subacute ADR Effects. The cardiomyopathy induced by chronic ADR treatment is characterized by the progressive dose-dependent degeneration of myocytes. To provide a biochemical basis for these observations, it is necessary to demonstrate progressive, dose-dependent biochemical changes in cell metabolism. The effect on RNA synthesis of multiple subacute double-injection courses of ADR treatment is presented in Table 2. Groups A, B, and C received 1, 2, and 3 courses, respectively, of ADR administered at 65-hr intervals. This time interval is sufficient for the inhibition of RNA synthesis to return to normal (Table 1). RNA precursor was again administered at the time of the last drug injection with sacrifice 17 hr later. This experiment demonstrated an increasing level of inhibition with increasing courses of drug treatment (p = 0.90 and 0.99). These results indicate either that RNA synthesis can be sensitized to inhibition by previous treatment or that transient recovery time is increased by multiple ADR doses. That the second possibility is reasonable is suggested by Group D. This group received 3 courses of ADR treatment as did Group C. However, RNA synthesis was not measured until the 48- to 65-hr interval after the final drug injection. At this time, RNA synthesis was still inhibited by 43% of control incorporation rather than returning to normal as observed following a single course of ADR treatment (Table 1). Alternatively, the results from Group D could represent a residual inhibition of RNA synthesis; *i.e.*, the rate of recovery of RNA synthesis following treatment may be constant but a portion, presumably dose dependent, of the RNA-synthesizing system could be irrevocably damaged.

In order to distinguish between a decreased rate of RNA recovery and residual effect due to multiple drug treatments, RNA synthesis was measured at 0, 48, 96, and 144 hr after

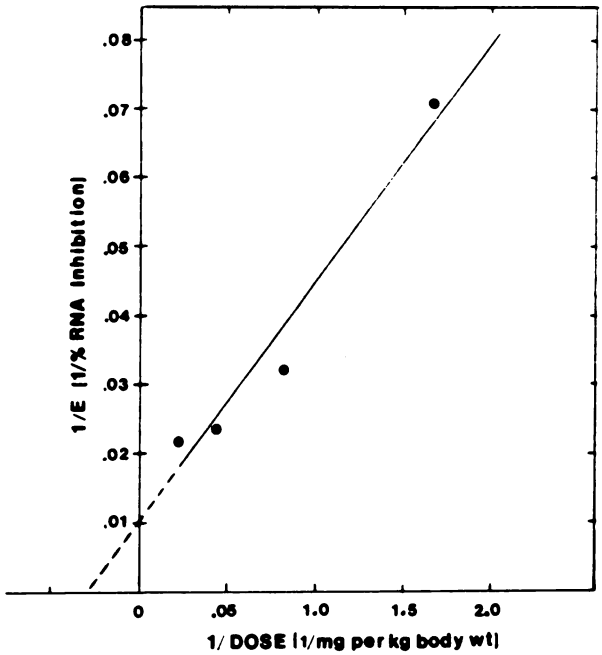

Chart 1. Effect of ADR dose on total myocardial RNA synthesis. Male Sprague-Dawley rats, in groups of 6 animals, were given 2 i.v. injections of ADR at the respective concentrations. Data are plotted as the reciprocals of the degree of RNA inhibition and amount of ADR (mg/kg body weight) administered. *Data points* were analyzed by linear regression. With the double reciprocal plot, the slope equals K_D/E_{max} (= 0.03), the y intercept equals $1/E_{max}$ (= 0.01), and the x intercept equals $1/K_D$ (= −0.29).

Table 1

RNA inhibition following single double-injection treatment with ADR

Treatment group (6/group)	Duration[a] of ADR exposure before radiolabeling (hr)	dpm RNA / A_{260} DNA[b]	% of inhibition	p[c]	dpm pool[d] / A_{260} DNA	p
Control	0	899 ± 122[e]			17,045 ± 2,223	
A	0	574 ± 23	30	0.99	17,908 ± 3,347	NS[f]
B	24	669 ± 64	18	0.95	18,266 ± 2,344	NS
C	48	867 ± 255	0	NS	16,942 ± 1,751	NS

[a] Radiolabeling was for 17 hr and occurred in the period immediately following the last ADR injection.
[b] Absorbance at 260 nm of hot PCA-soluble material from myocardial tissue.
[c] Statistical significance based on Student's t test.
[d] Cold PCA-soluble material from myocardial tissue.
[e] Mean ± S.D.
[f] NS, not significant.

Table 2

RNA inhibition following multiple subacute, double-injection courses of ADR

Treatment group (6/group)	Injection courses[a]	dpm RNA / A_{260} DNA[b]	% of inhibition	p[c]	dpm pool[d] / A_{260} DNA	p
Control	0	1,141 ± 146[e]			16,730 ± 4,357	
A	1	705 ± 87	38	0.99	15,155 ± 784	NS[f]
B	2	581 ± 143	49	0.99	14,659 ± 1,740	NS
C	3	435 ± 99	62	0.99	15,916 ± 2,131	NS
D	3	655 ± 94	43	0.99	15,256 ± 1,056	NS

[a] Double injection courses of ADR (1.25 mg/kg) were administered 1, 2, or 3 times at 65-hr intervals. RNA precursor was injected concurrent with the last ADR injection in Groups A, B, and C and 48 hr post-drug in Group D. Animals were sacrificed 17 hr later.
[b] Absorbance at 260 nm of hot PCA-soluble material from myocardial tissue.
[c] Statistical significance based on Student's t test.
[d] Cold PCA-soluble material from myocardial tissue.
[e] Mean ± S.D.
[f] NS, not significant.

the final ADR injection. Because of the multiple and extended course of these subacute drug treatment periods, a double-injection schedule of 1.25 mg ADR per kg body weight at 8-hr intervals was used. Each treatment group received 3 courses of double injections separated by 65-hr intervals. Radiolabeling again occurred over a 17-hr period. The results of this experiment (Chart 2*A*) demonstrate that, in fact, the inhibition of RNA synthesis remains transient and that the effect of multiple treatments is to increase the time required for complete recovery.

An alteration of RNA synthesis, as measured by uridine incorporation studies, may potentially reflect a change in RNA turnover. To examine this aspect of the problem, the rate of RNA degradation in control rats was compared to that in acute ADR-treated rats (Chart 3). For this analysis, 24 rats were submitted to the standard injection schedule for acute ADR treatment. Coincident with the second ADR injection, each animal received [^{3}H]uridine. A control group of 24 animals received radioisotope at the same time. At the times indicated in Chart 3, 3 control and 3 ADR-treated animals were sacrificed, and the specific radioactivity of the myocardial RNA was analyzed as described in "Materials and Methods." The data points obtained from 2 experiments were fitted to exponential equations (control $y = 1344e^{-0.0048}$, $r = 0.92$; treated $y = 1040^{-0.0056}$, $r = 0.88$). The half-life of the RNA from control animals was calculated to be 144 hr, and that from ADR-treated animals was 124 hr. This difference does not represent a significant alteration in RNA turnover rate due to ADR treatment.

Since the reduced rate of uridine incorporation by ADR reflects a true inhibition of RNA synthesis, it was of interest to the further understanding of the ADR-induced cardiomyopathy to probe the type of RNA that is inhibited. A first approximation was obtained by separating RNA into 2 classes: poly(A)+; and poly (A)−. This separation was achieved by affinity chromatography on oligodeoxythymidine cellulose (3). The separation of RNA into poly(A)+ and poly(A)− RNA generally reflects a differentiation between mRNA [poly(A)+] and rRNA [poly(A)−] (13). However, this distinction is not absolute since several mRNA species have been demonstrated to be devoid of the poly(A) sequence, most notably, the mRNAs for histones (1). RNA extracted from control and acute ADR-treated rats was analyzed by oligodeoxythymidine chromatography (Chart 4). Radioisotope was injected coincident with the second ADR treatment, and the animals were killed 17 hr later. The RNA from 4 control and 4 treated animals were pooled for analysis. The pooled fractions had the following specific radioactivities (dpm/A_{260} unit): poly(A)− of control, 826; poly(A)− of treated, 94; poly(A)+ of control, 2751; poly(A+) of treated, 346. Thus, both mRNA and rRNA are inhibited in ADR-treated rat hearts.

Chronic ADR Effects. The experiments presented describe the course of effects on RNA synthesis following acute and subacute ADR treatment. The multiple subacute ADR treatment

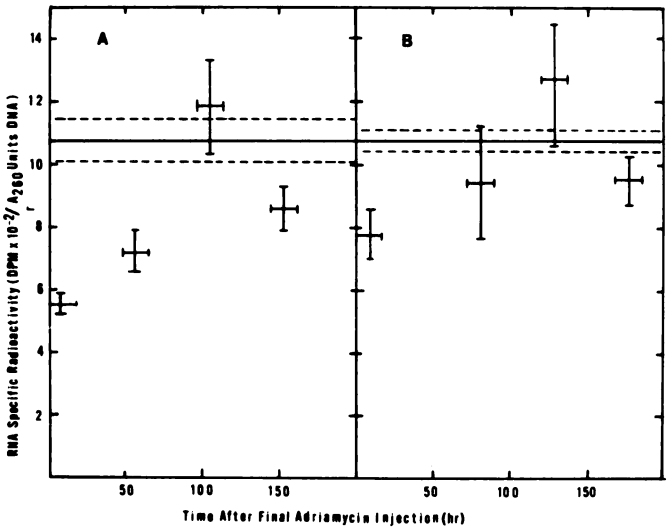

Chart 2. Transient inhibition of RNA synthesis following multiple and chronic ADR treatments. In *A*, 4 groups of 6 rats received 3 courses of double injections (1.25 mg/kg body weight) of ADR at 65-hr time intervals. [^{3}H]Uridine incorporation was measured 0, 48, 96, or 144 hr after the final course of ADR injections. In *B*, 4 groups of 6 rats received ADR (2.2 mg/kg body weight) once a week for 6 weeks. RNA synthesis was measured as above, but 0, 72, 120, 168 hr after the final ADR injection. *Horizontal limit lines*, 17-hr labeling period; *vertical limits*, S.E.; *solid horizontal line*, specific activity of the control group; *broken lines*, limits of the S.E.

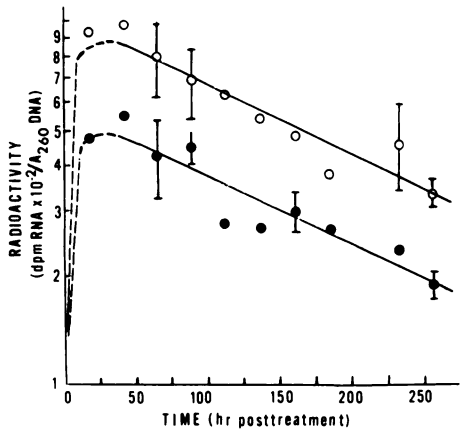

Chart 3. Kinetics of RNA synthesis and turnover. Rats, in groups of 6 animals, received a double-injection schedule (5 mg/kg) of ADR and were radiolabeled in the 17-hr period following the last ADR injection. At the times indicated, groups of treated and control animals were sacrificed. Data represent results from 2 separate experiments. ○, control; ●, treated. Identical time points in the 2 experiments are plotted as the mean; *bars*, S.E.

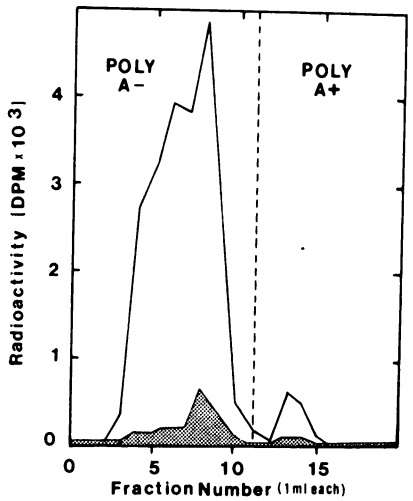

Chart 4. Inhibition of poly(A)+ RNA and poly(A)− RNA following acute ADR treatment. Two control and 2 ADR-treated rats (single-double-injection schedule) were sacrificed 17 hr postlabeling. Purified myocardial RNA was fractionated by oligodeoxythymidine affinity chromatography. *Dashed line* defines the change from binding buffer to elution buffer. *Stippled area*, RNA from treated animals.

schedule suggests that the effect of chronic ADR treatment upon RNA synthesis may be to extend the transient RNA inhibition. To test this hypothesis, animals were treated with 2.2 mg ADR per kg body weight once per week for 6 weeks. RNA synthesis was again measured, as in the subacute experiment, 0, 72, 120, and 168 hr after the final ADR injection (Chart 2*B*). These results confirm that following chronic treatment the inhibition of RNA synthesis by ADR is a transient effect and that, as in the multiple-course subacute experiment, the duration of the transient period is extended.

DISCUSSION

Most efforts to elucidate the mechanism of ADR cardiotoxicity deal with either the biochemical effect of single large doses or multiple "subacute" doses administered over periods of weeks to months. Although seldom stated, there appears to be a general belief that the 2 treatment modalities are unrelated in terms of biochemical effect. We have proceeded, however, under the assumption that chronic ADR treatment produces biochemical changes that differ from the acute changes only in magnitude. Therefore, in the present study, we have examined the relationship between the inhibition of RNA synthesis and various modalities of ADR treatment.

Our results demonstrate that inhibition of myocardial RNA synthesis by ADR is dose dependent (Chart 1) and that this inhibition is transient under all treatment conditions (Tables 1 and 2; Chart 2). Further, the duration of the period of transient inhibition of RNA synthesis is dose dependent (Table 1; Chart 2). The changes in RNA synthesis are not a result of changes in RNA turnover (Chart 3). Both mRNA and rRNA [as measured by poly(A) content] appear to be equally inhibited by ADR (Chart 4).

The interpretation of these data is relatively simple when considered in context with available pharmacokinetic data. Chart 5 approximates the cellular drug concentration and RNA synthesis as a function of time and the number of drug treatments. The pharmacokinetic data are based on the work of Siemann and Sutherland (15) and reflect their conclusions that: (*a*) the cellular concentration of ADR depends only on the dose and the amount of drug remaining from previous treatment; and (*b*) the pharmacokinetics of ADR is unaltered by both the amount and number of previous treatments.

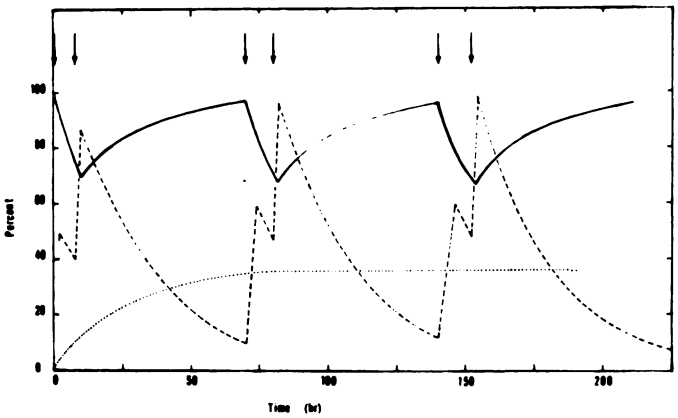

Chart 5. RNA synthesis relative to the kinetics of ADR uptake and degradation. Following mutliple injections of ADR (*arrows*), the kinetics (*dashed line*) of drug uptake and degradation has been calculated using the published value for $t_{1/2}$ of 20 hr (12). Assuming that the rate of RNA synthesis is inversely proportional to drug concentration at t_1, the kinetics of RNA synthesis is calculated (*solid line*). *Dotted line*, average drug concentration.

Following a single injection of ADR, the cellular drug level reaches a maximum in about 2 hr after which it decreases, through redistribution and biotransformation, at a constant rate ($t_{1/2}$ = 20 hr). With our conditions of acute treatment, a second injection of drug is administered after an 8-hr interval. The cellular drug concentration then becomes the sum of the amount of drug administered in the second dose and the amount of drug remaining at 8 hr from the first dose. Since the rate of RNA synthesis is inversely proportional to the cellular drug concentration, it would follow a course reciprocal to that observed for drug concentration. The transient inhibition of RNA synthesis reflects the sum of the rates of drug elimination. Similarly, subacute, multiple ADR treatment results in cellular ADR levels and concomitant degrees of RNA inhibition, dependent only on the dose currently administered and the amount of drug remaining from previous treatment(s).

These observations emphasize the importance of the dose and time dependency of the anthracycline cardiomyopathy. With the multiple subacute injection schedule, the inhibition of RNA synthesis is greater with each successive dose, which reflects the increasing intracellular drug concentration. Since the rate of drug turnover is unaltered by successive doses, the increasing drug level requires more time for biotransformation and consequently longer periods for recovery of RNA synthesis. Even at relatively low doses of drug administered at continuous short intervals, a definite finite amount of drug remains in the cell and, with successive injections, this level plateaus. Based on our data, the plateau value in subacute, multiple treatment conditions equals approximately 0.2 mg/kg body weight. The intracellular drug concentration should reach a plateau at about 80 hr.

These findings suggest that, at least under experimental conditions, frequent administration of maximal amounts of drug may elicit the most rapid ADR toxic effect in the heart. Utilizing intermittent ADR treatment schedules in cancer chemotherapy, the incidence of cardiomyopathy in humans is approximately 10 to 12% at total doses of greater than 500 to 600 mg/sq m (11). Under experimental conditions using maximal tolerated treatment doses administered continuously 2 to 3 times each week, the incidence of cardiomyopathy in rabbits after 10 to 11 weeks of treatment is greater than 75% at total doses of less than 300 mg/sq m (8).

In view of the variety of mechanisms proposed and the various biochemical effects having been shown to be elicited by anthracycline antibiotics, it is difficult at this time to incriminate any single effect as the definitive pathway responsible for the cardiomyopathy associated with chronic drug administration. Our studies confirm yet another effect of ADR which has previously been alluded to by other investigators (2, 19). The present findings of reduced cardiac RNA synthesis rates are supported by the findings of Zahringer *et al.* (18) who have demonstrated reduced myocardial total, messenger, and polyribosomal RNA in the ADR-treated rats. That these alterations in RNA metabolism have further consequences in cardiac cells is supported by the findings of Arena *et al.* (2) who have found impaired amino acid incorporation into myocardial protein of ADR-treated animals.

The fact that ADR alters DNA, RNA, and protein metabolism in a variety of cells is well documented in the literature (5, 17);

thus, it is not surprising that such an effect should occur in the heart. Recognizing that cardiac myocytes are mitotically static, it is reasonable to expect that DNA-dependent RNA and protein synthesis would be most notably affected in the heart. An imbalance between synthesis and degradation of myocardial RNA and protein could lead to a protracted loss of myocyte structural proteins. This suggestion is supported by the chronic, delayed nature of the anthracycline cardiomyopathy and the peculiar nature of the myocyte damage which is characterized not by the acute myonecrosis usually associated with toxins but rather by the protracted and insidious noninflammatory loss of myocyte organelles such as myofilaments and mitochondria from otherwise viable cells (9).

The data presented in this paper are insufficient at this time to suggest that altered RNA synthesis represents the definitive mechanism responsible for the anthracycline cardiomyopathy; however, our findings and those of others do suggest one reasonable explanation for this pathway. Further studies dealing with the synthesis of specific myocardial structural proteins such as individual myofilamentous or mitochondrial components may provide more definitive answers to this question.

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