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Alterations in DNA synthesis in cardiac tissue induced by adriamycin *in vivo*— Relationship to fatal toxicity

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Adriamycin (ADR) is a new antitumor antibiotic isolated from cultures of *Streptomyces peucetius var. caesius* [1]. ADR has rapidly become an important antitumor agent because of its activity against a wide range of solid tumors, and in particular, against a group of otherwise poorly responsive tumors, the soft tissue sarcomas [2] and sarcomas of bone [3, 4]. Enthusiasm for ADR has been tempered, however, as anthracycline antibiotics, such as ADR, possess dose-limiting, fatal, cardiac toxicity. Animal models, while said to be capable of predicting acute bone marrow toxicity [5], failed until recently [6, 7] to demonstrate delayed cardiac toxicity. As ADR has been shown to inhibit DNA as well as RNA synthesis [8], we studied the pattern of suppression and recovery of DNA synthesis, as reflected in the incorporation of tritiated thymidine (^{3}H-TdR) into DNA, in the bone marrow (BM) and gastrointestinal mucosa (GI), and in the important site of unusual toxicity, the cardiac muscle.

Popular conception suggests that little if any DNA synthesis occurs in the adult myocardium. As an example, Zak [9], in a review of cell proliferation during cardiac growth, indicated that heart muscle cells divide only during the first 3 weeks of postnatal life. Claycomb [10] reported that DNA synthesis in rat cardiac muscle is essentially "turned off" by day 17 of postnatal development. Nevertheless, there is considerable information suggesting that, in fact, low levels of DNA synthesis do persist in adult myocardial tissue. Actually, in Claycomb's studies, a basal low level of DNA synthesis in the adult heart did persist, as measured by ^{3}H-TdR incorporation into DNA. Furthermore, studies by Petersen and Baserga [11] have demonstrated a two-phase growth pattern of the ventricular myocardium in mice. The first phase during early postnatal life (0 to 5 weeks old) is associated with an increase in both number and size of myocardial cells, and the second, occurring in adult mice (after 5 weeks of age), is associated with an increase in the size of myocardial cells and a moderate increase in the number of nuclei in the ventricles

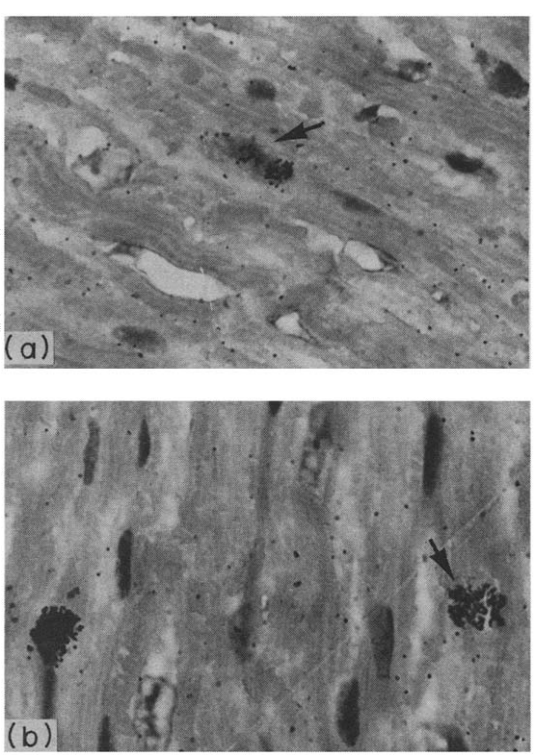

Fig. 1. Autoradiograph of the myocardium of a 10-week-old mouse following 24 hr of pulse labeling *in vivo* with ^{3}H-thymidine showing (A) a labeled cardiac muscle cell nucleus (arrow) and (B) a mitotic figure (arrow) in the background of the cytoplasm of cardiac muscle (H × E) (× 880).

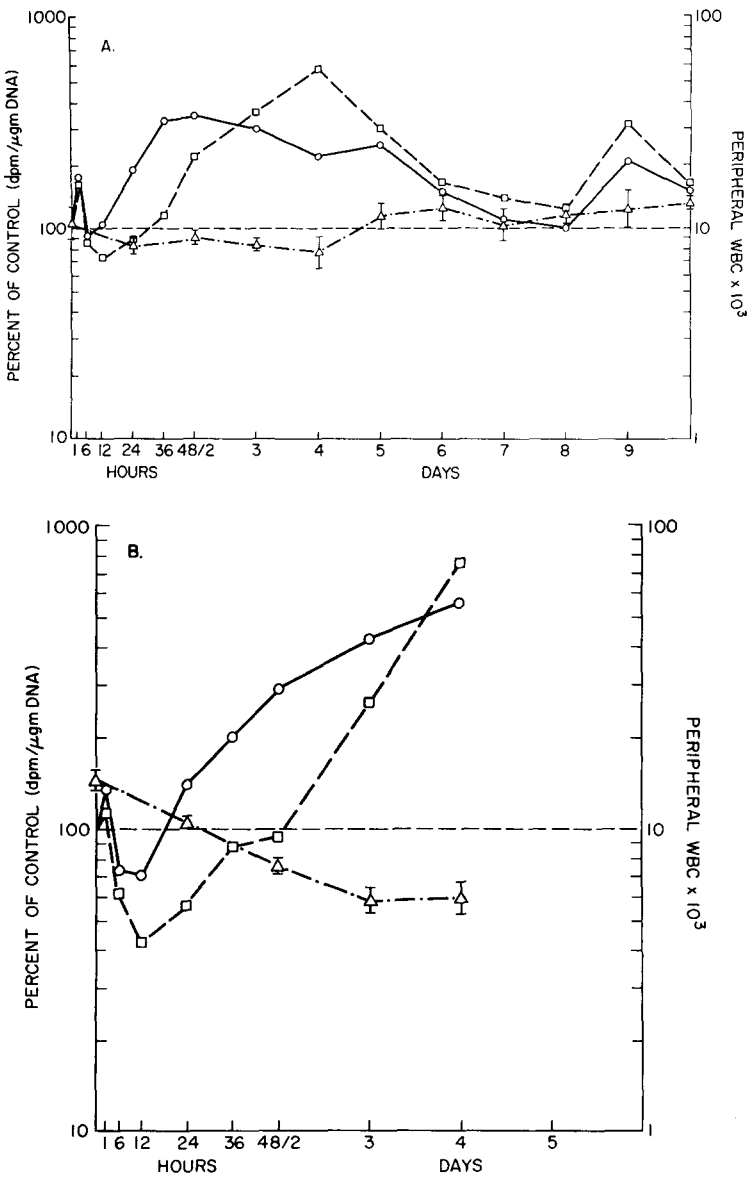

Fig. 2. Effect of a single dose of adriamycin (ADR) on the incorporation *in vivo* of ^{3}H-thymidine into DNA of normal murine bone marrow and gastrointestinal mucosal cells and on the peripheral white blood count (WBC): (A) ADR, 10 mg/kg, i.p., and (B) ADR, 20 mg/kg, i.p. Each point represents the mean of two pooled groups of three animals each. Values are expressed as the per cent of control of the dis./min/μg of DNA for each of the two separate tissues. Control values $\pm$ the standard error are as follows: bone marrow, 866 $\pm$ 48 dis./min/μg of DNA, and gastrointestinal mucosa, 855 $\pm$ 90 dis./min/μg of DNA. The peripheral WBC is graphed as the mean of six individual determinations at each time point $\pm$ the standard error. Key: O——O, bone marrow; □---□, gastrointestinal mucosa; and △-·-△, peripheral WBC.

of the heart. Nevertheless, both phases are associated with some DNA synthesis, as measured by ^{3}H-TdR incorporation into myocardial cells, although during the second phase this is markedly reduced. More recently, increased DNA synthesis has been demonstrated in the myocardium of rats in response to induced renal hypertension [12]. It is clear, therefore, that continuous low levels of DNA synthesis do occur in adult myocardium, and the level of ^{3}H-TdR incorporation may be altered by physiologic alterations of the myocardium. In the present study, the mean baseline incorporation of ^{3}H-TdR into DNA of cardiac muscle of 2 to 3-month-old mice was 221 $\pm$ 31 dis./min/μg of DNA for twelve determinations involving 36 mice. While it is only 23 per cent of that usually observed in two of the hosts' more rapidly proliferating tissues, the BM and GI, it is consistent and reproducible.

BDF_1 female mice, average weight 20 g, were used throughout these experiments. DNA synthesis was studied as reflected in the incorporation of ^{3}H-TdR for ADR studies or tritiated deoxyuridine (^{3}H-UdR) for the studies with 5-fluorouracil (5-FU). Six mice were left untreated and served as the 0 hr control. After intraperitoneal (i.p.) chemotherapy, at time 0 and at various times as indicated, six mice at each time point received 100 μCi ^{3}H-TdR (sp. act. 6·7 Ci/m-mole) i.p. or 100 μCi ^{3}H-UdR (sp. act. 12·4 Ci/m-mole) i.p. and 1 hr later were sacrificed. BM and GI were collected as described in previous publications [13, 14]. The hearts were removed, minced, crushed using

a nylon-type tissue grinder, and suspended in iced phosphate-buffered saline. Separate samples of heart, GI and BM from each of six mice were pooled into two groups of three. Pooled samples were centrifuged at 800 *g* for 5 min at 4°. The button of tissue from the pooled specimens was extracted by a modification of the procedure of Schneider [15] for determination of DNA content and included an alkaline hydrolysis to remove RNA [16]. An aliquot of the final supernatant was added to liquid scintillation fluid (LSF) and counted in a liquid scintillation spectrometer. A standard quench curve for tritium in LSF was used for the determination of quenching error. An aliquot of the final supernatant was processed by the method of Burton [17] for DNA determination. Results were expressed as the dis./min/μg of DNA graphed as per cent control over time. Control data (pretreatment) used in these studies $\pm$ the standard error are as follows: (a) ^{3}H-TdR studies: BM, 866 $\pm$ 48 dis./min/μg of DNA; GI, 855 $\pm$ 90 dis./min/μg of DNA; and cardiac muscle, 221 $\pm$ 31 dis./min/μg of DNA; and (b) ^{3}H-UdR studies: BM, 831 $\pm$ 124 dis./min/μg of DNA; GI mucosa, 934 $\pm$ 152 dis./min/μg of DNA; and cardiac muscle, 152 $\pm$ 17 dis./min/μg of DNA. Peripheral white blood counts (WBC) were performed on venous blood samples of six mice at each time point and were expressed as the mean $\pm$ standard error.

Autoradiography was performed on normal mice after receiving 100 μCi ^{3}H-TdR (17·6 Ci/m-mole; New England Nuclear, Boston, Mass.) i.p. at 6-hr intervals for four injections. The hearts were removed, ventricular cross sections fixed in 10% formalin and pressed for paraffin embedding. Sections were cut at 4 μ, stained with eosin, dipped in Kodak NTB3 emulsion, exposed for 14 days at 4°, developed, fixed, and stained with hematoxylin.

Figure 1A shows a labeled nucleus in cardiac muscle and Fig. 1B indicates a mitotic figure as well as a labeled nucleus. In sections not shown, labeled endothelial and interstitial cell nuclei were also seen.

Figure 2A shows the effect of 10 mg/kg of ADR (LD_{30-50}) upon DNA synthesis as reflected in the incorporation of ^{3}H-TdR into DNA in BM and GI of normal mice. Only a marginal depression below control levels, 92 and 86 per cent of control, respectively, is seen. The peripheral WBC showed no significant fall. Figure 2B shows the effect of a single dose of 20 mg/kg of ADR (LD_{80-90}) upon DNA synthesis again as reflected in the incorporation of ^{3}H-TdR into the DNA in the same two tissues. Again there is a short-lived (< 24 hr) mild depression of DNA synthesis in the BM ($\simeq$ 70 per cent of control) with a nadir peripheral WBC of only 6 $\times$ 10^3 cells/mm^3. Neither the mild, short-lived depression of DNA synthesis in normal host rapidly proliferating tissues (BM and GI), nor the modest fall in peripheral WBC at the higher dose could account for the lethal toxicity manifest by these doses of ADR.

As the cardiac toxicity was a clinically important side-effect of anthracycline antibiotics in man [18], we explored the possibility that cardiac toxicity might be dose limiting in these mice and might be mirrored by suppression of DNA synthesis in cardiac tissue. Figure 3A and B shows the effect of ADR (10 mg/kg and 20 mg/kg) upon DNA synthesis, as reflected in the incorporation of ^{3}H-TdR in the DNA in the heart. The survival of mice at each dose is graphed below. A single dose of 10 mg/kg of ADR (LD_{30-50}) (Fig. 3A) produced an early, mild depression of DNA synthesis in the cardiac muscle which returned to control by 12 hr, and was followed by a delayed depression of DNA synthesis in the heart (< 35 per cent of control) which was temporally related to fatal toxicity. This depression is more marked at the more toxic dose of ADR (20 mg/kg), an LD_{80-90} (Fig. 3B). Once again a modest early depression of DNA synthesis, not accompanied by fatal toxicity, is followed by a delayed, profound depression of DNA synthesis in cardiac muscle (< 15 per cent of control) which precedes and is continued through the period of increasing mortality.

As a test of the specificity of this measure of cardiac injury we studied the effect upon DNA synthesis as reflected in the incorporation of ^{3}H-UdR into DNA in cardiac tissue of an equitoxic dose (LD_{80}) of a classical antimetabolite known to markedly depress DNA synthesis in

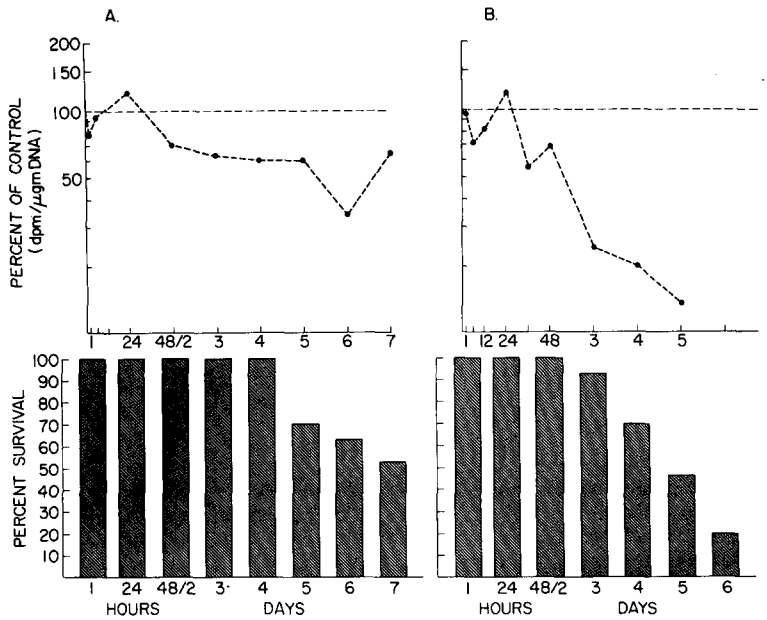

Fig. 3. Effect of a single dose of ADR on the incorporation *in vivo* of ^{3}H-thymidine into DNA of cardiac muscle of normal mice and on the survival of normal mice: (A) ADR, 10 mg/kg, i.p., and (B) ADR, 20 mg/kg, i.p. Each point represents the mean of two pooled groups of three animals each. Values are expressed as the per cent control of the dis./min/μg of DNA. Control values for the cardiac muscle $\pm$ the standard error were 221 $\pm$ 31 dis./min/μg of DNA. Survival bars represent the per cent of the initial 30 animals alive each day after a single dose of ADR: (A) 10 mg/kg, i.p., and (B) 20 mg/kg, i.p. Key: ●----●, heart.

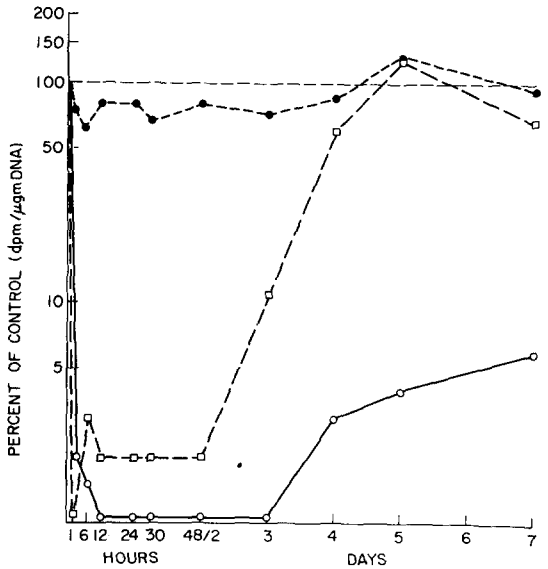

Fig. 4. Effect of a single dose of 400 mg/kg, i.p., of 5-fluorouracil on the incorporation *in vivo* of ^{3}H-deoxyuridine into DNA of normal murine bone marrow, gastrointestinal mucosa and cardiac muscle. Each point represents the mean of two pooled groups of three animals each. Values are expressed as per cent of control of the dis./min/μg of DNA for each of the three separate tissues. Control values ± the standard error are as follows: bone marrow, 831 ± 124 dis./min/μg of DNA; gastrointestinal mucosa, 934 ± 152 dis./min/μg of DNA; and cardiac muscle, 152 ± 17 dis./min/μg of DNA. Key: O——O, bone marrow; □----□, gastrointestinal mucosa; and ●----●, cardiac muscle.

the rapidly proliferating host target tissues, but not known to possess any cardiac toxicity [19]. Figure 4 shows the effect of a single dose of 400 mg/kg of 5-FU upon DNA synthesis in the BM, GI and heart. A rapid, marked depression of DNA synthesis to 1 per cent of control was affected in the rapidly proliferating host target tissues, BM and GI. However, this equitoxic dose (LD_{80}) of 5-FU, which possesses no known cardiac toxicity, did not markedly depress DNA synthesis in cardiac muscle. For comparison, on day 5 after 5-FU, DNA synthesis in the heart is approximately 150 per cent of control, whereas on the same day after an equitoxic dose of ADR (20 mg/kg), DNA synthesis in the heart is depressed below 15 per cent of control. This study shows that inhibition of DNA synthesis in the heart is not a non-specific effect of lethal injury, but at least in the mouse, ADR demonstrates a major disruption of DNA synthesis in cardiac tissue.

Our findings of baseline levels of DNA synthesis in mouse cardiac muscle (Figs. 1 and 3) are in accord with those of Petersen and Baserga [11] as well as Pelc [20], who noted that the thymidine-labeling index of mouse heart muscle, as well as those of other well differentiated tissues, was greater than could be explained by mitotic activity. Pelc suggested that the newly formed DNA represents "metabolic DNA," i.e. self-replicating, short segments of DNA which can regulate and perform the transcription of mRNA. Other possible explanations such as DNA repair or mitochondrial DNA synthesis might also explain this low level of DNA synthesis in the adult myocardium.

In addition to the alterations seen in DNA synthesis, histologic sections of mouse hearts stained with hematoxylin-eosin after a single dose of 15 mg/kg of ADR showed myocytolytic changes, similar to those described in patients with daunomycin-induced cardiomyopathy [21]. Characteristic light and electron microscopic alterations similar to the anthracycline-induced cardiomyopathy seen in man have been seen in this mouse model.* Particular attention was directed to the vascular and endothelial areas as well as the cardiac interstitial cells, as Petersen and Baserga [11] had indicated that DNA synthesis in the adult mouse was limited to endocardial, endothelial and connective tissue cells. Nevertheless, no vascular or endothelial damage or inflammation was noted at the time of light microscopic examination of our cardiac specimens.

Whether or not DNA synthesis observed in cardiac muscle represents continuous DNA repair, motochondrial DNA synthesis, "metabolic DNA," or DNA synthesis in endothelial and interstitial cells, the ADR-induced delayed depression of DNA synthesis in cardiac muscle is provocative and may reflect disruption of vital vascular, or metabolic processes required to maintain the electromechanical function of the heart. Such a disruption could be life-threatening. An alternative postulate is that the disruption of DNA synthesis is not a crucial primary feature of the cardiac toxicity of ADR, but merely reflects the cardiac injury. Even if this is the case, the study of suppression of DNA synthesis in cardiac tissue may provide a technique for defining the cardiac toxicity of this important antitumor agent and for testing potential new anthracycline antibiotics which have been derived to minimize cardiac toxicity.

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