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Reduction of Acute Adriamycin Toxicity in Mice Treated with Adenosine*

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Abstract—*The effects of adenosine on the acute toxicity and oncolytic activity of adriamycin (ADR) were evaluated in mice. When administered as a single i.p. injection of* 17.5 mg/kg, *adriamycin produced death in all mice within* 12 days *after treatment, with a mean survival time of* 5–9 days. *In contrast, the mean survival time of mice administered adenosine subcutaneously* (200 mg/kg) *in addition to adriamycin was significantly increased compared to adriamycin-treated mice. The protection elicited by adenosine was apparently not a generalized phenomenon of purines, however, since neither hypoxanthine nor inosine were effective protectants. Although a number of adenosine treatment schedules were tested, it was found that adenosine given immediately after adriamycin was as effective as multiple adenosine injections. Administration of adenosine had no apparent effect on adriamycin-mediated changes in ventricular weight, leukocyte count, elevated serum lactic dehydrogenase (LDH) activity or in the histopathologic changes observed in selected tissues. Two grossly observable effects of adenosine administration were lethargy and peripheral hypothermia, which were first noticed approximately* 15 min *after adenosine administration and which lasted for up to* 2 hr. *Finally, adenosine had no adverse effect on the antitumor efficacy of adriamycin against L*1210 *ascites cells inoculated i.p. to* BDF_1 *mice.*

INTRODUCTION

THE ANTHRACYCLINE antibiotic adriamycin (ADR)[3] has significant antitumor activity against a wide range of human malignant neoplasms [1]. Unfortunately, the clinical usefulness of this important antitumor compound is hampered by its propensity to produce a severe, and sometimes life-threatening, cardiomyopathy in patients administered a cumulative dose of ADR exceeding 550 mg/m^2 [2]. For this reason, intensive efforts have been expended to maintain the antitumor activity of ADR while decreasing its cardiotoxicity. To date, these efforts have included the development of less cardiotoxic anthracycline derivatives, altered treatment schedules and the study of various pharmacologic interventions to prevent anthracycline-mediated cardiomyopathy.

Although anthracycline analogs such as 4′-*0*-tetrahydropyranyl adriamycin [3] and AD-32 [4] have been reported to produce less cardiotoxicity than ADR, the development of non-cardiotoxic anthracyclines has met with limited success. Benjamin *et al.* [5] have recently reported that intermittent low-dose ADR infusion produced less cardiac damage than standard ADR treatment regimens, with no significant decrease in the oncolytic activity of ADR. This observation requires further evaluation before the utility of such an approach can be fully assessed. A number of pharmacologic agents, including ICRF-187 [6], coenzyme Q_{10} [7], *N*-acetylcysteine [8], vitamin E [9] and ascorbic acid [10] have been reported to inhibit or at least diminish selected ADR-induced biochemical perturbations and thereby decrease ADR toxicity *in vivo*.

Few, if any, of these studies, however, have demonstrated significant 'protection' in both *in vitro* and *in vivo* test systems. Recently, we [11] and others [12] have demonstrated that adenosine blocks the toxic effects of ADR on isolated rat myocardial cells. Seyradarian and Artaza have suggested that the adenosine cardioprotection

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may have resulted from a maintenance of intracellular ATP concentration and adenylate charge, which have been shown to be decreased by ADR [12]. The present studies were undertaken to determine whether these *in vitro* observations could be extended to the whole animal.

MATERIALS AND METHODS

Male CD_1 mice (20–25 g) were purchased from Canadian Breeders Laboratories, Montreal, Quebec. Male C57BL/6 × DBA/2 mice (hereafter referred to as BDF_1 mice) were obtained from Jackson Breeding Laboratories, Bar Harbor, ME. All animals were housed in our central animal facilities that maintain a constant temperature, relative humidity and photoperiod. Food and water were available *ad libitum*. Adriamycin hydrochloride was a generous gift from Adria Laboratories (Columbus, OH), adenosine was purchased from Sigma Chemical Co. (St. Louis, MO) and enzyme assay kits for the determination of serum lactic dehydrogenase (LDH) activity were obtained from Calbiochem (La Jolla, CA). Data were analyzed for statistical significance using Student's t test, with $P < 0.05$ chosen as the level of significance.

Effect of adenosine on the acute toxicity of ADR in CD_1 mice

Solutions of ADR and adenosine were prepared immediately prior to use by dissolving the compounds in sterile double-distilled water and sterile 0.9% NaCl respectively. ADR was administered as a single i.p. injection and adenosine was administered subcutaneously. Following treatment, mice were observed daily for evidence of toxicity. In selected studies, peripheral blood was taken from the retro-orbital sinus of mice for enumeration of leukocytes and the measurement of serum LDH concentration. At the appropriate times, mice were then killed by cervical dislocation and the hearts excised. After careful removal of the auricles and other extraneous tissue, wet ventricular weights were measured. The ventricles, liver, spleen, kidney, duodenum, brain and femoral bone marrow were then fixed in 10% neutral buffered formalin for histopathologic examination.

Effect of adenosine on the antitumor activity of ADR in mice bearing L1210 leukemia cells

BDF_1 mice were inoculated i.p. with 10^6 ascites L1210 murine leukemia cells. Twenty-four hours later, ADR (13.3 mg/kg) was administered as a single i.p. injection immediately prior to a single subcutaneous injection of adenosine (200 mg/kg) or 0.9% NaCl (0.1 ml/10 g of body weight). Control mice were administered 0.9% NaCl i.p. immediately prior to a subcutaneous injection of either adenosine or 0.9% NaCl. Animals were observed daily for evidence of toxicity and survival. The mean survival time (MST) was calculated for each treatment group and the efficacy of each regimen determined using the following formula:

$$\text{Percentage increased left span (ILS)} = \left(\frac{\text{MST}_{\text{treated}}}{\text{MST}_{\text{control}}}\right) - 1 \times 100.$$

RESULTS

Effect of adenosine on the acute toxicity of ADR in non-tumor-bearing mice

CD_1 mice were administered 200 mg adenosine/kg body weight s.c. 1, 12 and 24 hr preceding and following a single i.p. injection of 17.5 mg ADR/kg. As shown in Fig. 1, mice administered adenosine in combination with ADR had a significantly increased mean survival time (25 ± 7.2 days) compared to mice administered ADR alone (5.4 ± 1.5 days). In addition, whereas all mice treated with ADR alone died within 12 days, 2 of 8 animals administered ADR and adenosine were alive 30 days after treatment. In contrast, neither hypoxanthine nor inosine had any effect on the acute toxicity of ADR when administered in an identical regimen as adenosine (data not shown).

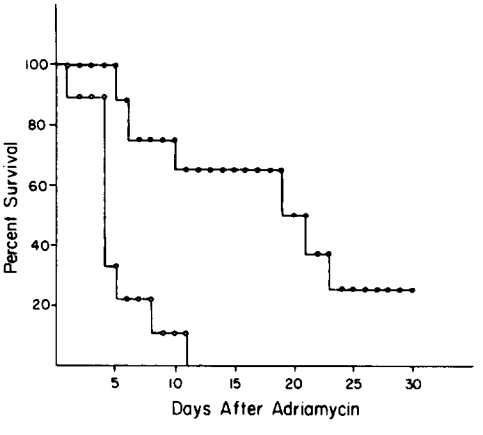

Fig. 1. Survival data for mice administered either adriamycin (O——O) or adriamycin plus adenosine (●——●). CD_1 mice were treated with either adriamycin (17.5 mg/kg, i.p.) or adenosine (200 mg/kg, s.c.) 24, 12 and 1 hr prior to and following a single i.p. injection of adriamycin.

The reduction of ADR toxicity by adenosine was further substantiated by the differences in body weight observed in the two treatment groups (Fig. 2). Mice administered ADR alone lost weight continually following treatment. In contrast, mice administered ADR in combination with adenosine lost weight for the first 4 days following

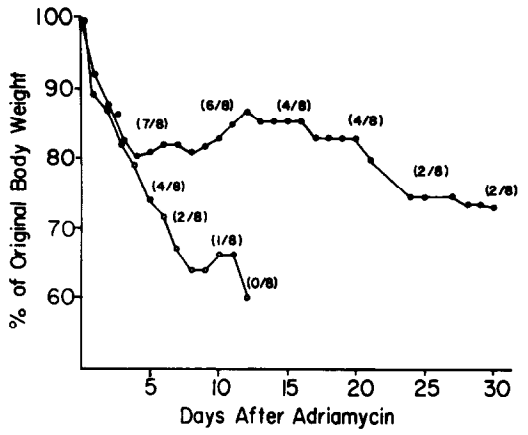

Fig. 2. Body weight loss of mice administered either adriamycin (○——○) or adenosine plus adriamycin (●——●). Numbers in parentheses represent surviving mice/treated mice. For experimental procedure see Fig. 1.

treatment. Thereafter, however, their body weight remained relatively constant until day 20, at which time a secondary loss in body weight was observed. Mice treated with either 0.9% NaCl or adenosine alone gained weight throughout the observation period.

A consistent observation made throughout these studies was that mice administered adenosine by itself or in combination with ADR became extremely lethargic within 15 min after adenosine administration. This lethargy persisted for approximately 2 hr, after which time no residual effects were apparent. Although body temperatures were not measured, an obvious peripheral hypothermia was noted in mice displaying the adenosine-induced lethargy.

Because of the significant lethargy and hypothermia induced by adenosine administration, it was of interest to determine whether the number of adenosine injections or actual dose of adenosine could be reduced and still diminish ADR toxicity. To do this, two experiments were performed. Initially, mice were administered 200 mg adenosine/kg at several different times with respect to ADR. As depicted in Table 1,

Table 1. Effect of treatment schedule on adenosine (ADO) reduction of adriamycin (ADR) toxicity

Treatment*	ADO schedule	Mean survival time (days)
ADR	—	5.5 ± 1.0
ADR + ADO	-24, -12, -1, +1, +12, +24	22.5 ± 5.5†
ADR + ADO	-1, +2	19.3 ± 4.8†
ADR + ADO	simultaneously	21.5 ± 8.1†

*Mice were administered ADR (17.5 mg/kg) i.p. and ADO (200 mg/kg) subcutaneously at various times (hr) with respect to ADR.

†Mean survival times (± 1 S.E.) are significantly different ($P<0.05$) from controls by Student's t test.

adenosine was equally effective regardless of the treatment schedule tested. Since a single s.c. injection of adenosine (200 mg/kg) administered immediately following a single i.p. injection of ADR (17.5 mg/kg) significantly reduced acute toxicity of ADR, it was of interest to determine the most effective dose of adenosine. Mice were administered a single s.c. injection of either 400, 200 or 100 mg adenosine/kg immediately following a single i.p. injection of 17.5 mg ADR/kg. All 6 mice administered ADR alone died within 15 days of treatment, with an MST of 9.3 ± 2.4 days. In contrast, 50% of mice administered either 400 or 200 mg adenosine/kg and 33% of mice administered 100 mg adenosine/kg were alive 30 days after ADR treatment (Table 2).

In an attempt to identify a possible mechanism of adenosine protection, mice were administered 200 mg adenosine/kg s.c. immediately following a single i.p. injection of 17.5 mg ADR/kg. Four days later, mice were bled for determination of leukocyte count and LDH activity. Mice were then killed, ventricular muscle wet weight measured and tissues taken for histopathologic examination. ADR treatment produced significant leukocytopenia and elevated serum LDH activities which were not altered by administration of concomitant adenosine (Table 3). Light microscopy revealed desquamation of the epithelial

Table 2. Effect of adenosine dose on the acute toxicity of adriamycin

Treatment*			Long-term survivors†
Adenosine (mg/kg)	Adriamycin (mg/kg)	Mean survival time (days ± 1 S.E.)	(survivors/treated)
0	17.5	9.3 ± 2.4	0/6
100	17.5	21.7 ± 2.9	2/6
200	17.5	24.7 ± 2.7	3/6
400	17.5	25.1 ± 2.4	3/6

*Adenosine was administered as a single subcutaneous injection immediately following a single i.p. injection of adriamycin.

†Animals alive 30 days after adriamycin administration were considered to be long-term survivors.

Table 3. Lack of adenosine (ADO) effect on selected parameters of adriamycin (ADR) acute toxicity

Treatment*	Ventricle weight (mg/100 g body wt)	Leukocyte number (× 16/ml)	LDH (mU/ml)
0.9% NaCl	5.10 ± 0.47	4.40 ± 0.6	116 ± 40
ADO	4.90 ± 0.46	4.60 ± 0.5	157 ± 20
ADR	4.40 ± 0.26	0.75 ± 0.3†	2074 ± 445†
ADR + ADO	4.47 ± 0.29	1.25 ± 0.6†	1896 ± 543†

*Adriamycin (17.5 mg/kg, i.p.) and/or adenosine (200 mg/kg, s.c.) were administered to mice simultaneously.

†Values (mean ± 1 S.E., $n = 6$) are significantly different ($P < 0.05$) from 0.9% NaCl- or adenosine-treated mice.

lining, edema and hemorrhage in the duodenum, as well as hypocellular bone marrow in mice treated with either ADR or ADR plus adenosine. Surprisingly, no treatment-related changes were observed in hearts taken from either treatment group.

Effect of adenosine on the antitumor activity of ADR in mice bearing L1210 leukemia cells

The preceding experiments clearly demonstrate that adenosine significantly decreased the acute toxicity of i.p.-administered ADR. It was necessary, however, to also assess the effect of adenosine on the oncolytic activity of ADR. To do this, BDF_1 mice were inoculated i.p. with 10^6 L1210 leukemia cells and were administered either 0.9% NaCl, ADR (13.3 mg/kg), adenosine (200 mg/kg) or adenosine plus ADR 24 hr after tumor inoculation. As shown in Fig. 3, no significant difference was observed in the MST of mice treated with either ADR or ADR plus adenosine, nor did adenosine have any intrinsic oncolytic activity in this tumor system.

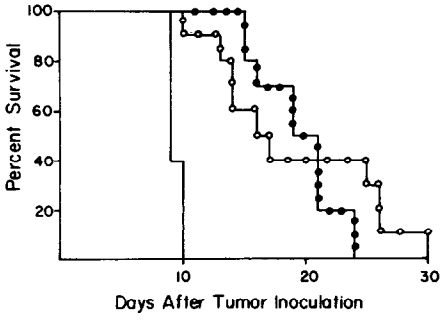

Fig. 3. Effect of adriamycin (○——○) or adenosine plus adriamycin (●——●) on the survival time of leukemic BDF_1 mice. Mice were inoculated i.p. with 10^6 L1210 murine leukemia cells and 24 hr later treated with either adriamycin (13.3 mg/kg, i.p.) or adenosine (200 mg/kg, s.c.) immediately following a single i.p. injection of adriamycin (13.3 mg/kg).

DISCUSSION

Cardiomyopathy is a serious dose-limiting complication encountered in patients administered ADR. Although the mechanism remains unclear, a number of hypotheses have been proposed which include altered membrane fluidity [13], intermediary glucose metabolism [14], calcium ion concentration [15], inhibition of Na–K ATPase activity [16] and lipid peroxidation [17]. Similarly, a wide variety of putative myocardial protectants, such as coenzyme Q_{10} [7], vitamin E [9], *N*-acetylcysteine [8] and ascorbic acid [10], have been investigated. Unfortunately, these compounds have had limited success in selectively preventing ADR-mediated cardiotoxicity.

Recently, we [11] and others [12] have reported that adenosine effectively prevented ADR-induced changes in isolated myocardial cells from rat ventricles. The present work was undertaken to determine whether a similar decrease in ADR toxicity would be observed in whole animals. The results of these studies demonstrate that adenosine significantly diminished the acute toxicity of ADR administered as a single i.p. injection without adversely affecting the antitumor activity of ADR. Further, these studies suggest that the protection was not common to all purines but, rather, specific for adenosine. Mice that survived the acute toxic effects of ADR, however, subsequently developed a delayed toxicity and began dying approximately 20 days after ADR administration. Similar results have been reported by others using such protective agents as vitamin E [9], coenzyme Q_{10} [7] and ascorbic acid [10].

Although the mechanism of the delayed toxicity is poorly understood, it has been suggested that the toxicity may result from a generalized peritonitis induced by i.p. administration of ADR. The argument for non-specific peritonitis as the cause of delayed toxicity observed in mice treated i.p. with ADR is strengthened by the fact that no such sequelae are observed when ADR is administered i.v. [18]. Further, VM 26, an epipodophyllotoxin with some structural similarity to ADR, also produced a delayed toxicity when administered i.p. but not when administered i.v. [19, 20]. More recently, it

has been reported that aclacinomycin, a dehydroxylated derivative of ADR, produced no delayed toxicity in i.p.-treated mice [21]. These investigators propose that it is the presence of the parahydroxyl groups adjacent to the quinone moieties on both ADR and VM 26 that is responsible for the delayed toxicity.

Elucidation of the actual mechanism(s) of decreased ADR toxicity by adenosine is complicated by the large number of physiological and biochemical effects produced by adenosine in the whole animal. For example, adenosine has been reported to alter ATP generation [22], interact with purinergic receptors [23], relax vascular smooth muscle [24] and inhibit cyclic AMP accumulation following norepinephrine stimulation of lipocytes [25], but to increase cyclic AMP concentration in a number of other tissues [26–28].

Adenosine has been demonstrated conclusively to increase coronary blood flow [29, 30]. Since many of the acute effects of ADR on the heart are quite similar to those observed in ischemia-damaged hearts and Herman *et al.* [31] have shown that ADR significantly decreased coronary blood flow of isolated dog hearts, it is possible that exogenously administered adenosine may be acting through coronary vasodilation. This increased coronary blood could, therefore, counteract the adverse effects of ADR on the coronary vasculature and thereby diminish ischemic damage.

Another possible explanation for the interaction between ADR and adenosine may rest in the fact that subcutaneously administered adenosine produced profound lethargy and hypothermia in mice. Recently, Gailis reported that chlorpromazine also decreased the acute toxicity of ADR [32]. He proposed that a possible mechanism of this protection was through the hypothermia induced by chlorpromazine. This hypothesis is strengthened by recent clinical studies demonstrating that localized hypothermia applied to the scalp of patients greatly decreased the incidence of ADR-induced alopecia. Further, this effect on alopecia could not be solely explained by altered drug distribution, since little difference in tissue levels of ADR were detected in cooled areas. Thus it is possible that adenosine may also protect the mouse by producing systemic hypothermia.

In summary, the present report demonstrates that adenosine can significantly prolong the survival time of mice treated with ADR i.p. without adversely affecting the oncolytic activity of ADR. This protective effect of adenosine is in agreement with previous reports demonstrating that adenosine also diminished the toxic effects of ADR in isolated myocardial cells.

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