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Adriamycin and Daunomycin Dose-dependent Effects Upon Contractions of Isolated Rat Myocytes

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Summary. *Chemotherapeutic usefulness of adriamycin (ADR) and daunomycin (DAU), members of a large class of antitumor anthracyclines, is limited by a unique cardiotoxicity. Using spontaneously beating isolated myocytes from adult rat hearts, we have observed a relatively unique effect of these agents upon maximal contraction times. ADR and DAU induce cessation of beating in an identical dose-dependent manner, while two related anthracyclines exhibit similar inhibitory effects but at different concentrations. Other cytotoxic and antitumor agents tested failed to significantly affect maximal contraction times. This system may be useful in the evaluation of anthracycline analogs for cardiotoxic potential relative to ADR and DAU, as well as in studying the mechanisms of that toxicity. It may also prove useful in selective examination of the direct effects of other agents upon myocardial cells.*

Introduction

Despite numerous reports of the cardiotoxicity of the antitumor antibiotics adriamycin (ADR) and daunomycin (DAU) [2, 9, 15], the mechanism by which they produce life-threatening myocardial lesions is not yet understood. Cardiac damage may not become apparent until months after cessation of treatment, at which time a patient may rapidly develop intractable congestive heart failure [13, 24]. There is as yet no acute method that allows human cardiotoxicity of these drugs to be reliably predicted. Nor does there appear to be a rapid and accurate system for evaluating the potential cardiotoxicities of drugs similar to ADR and DAU.

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In the Developmental Therapeutics Program of the National Cancer Institute there exists antitumor information on over 550 analogs of ADR. Although approximately one-third of the analogs have antitumor activities warranting further investigations, the cardiotoxicities of most have not been determined because the current rabbit histopathology screen takes over 6 months to perform [8].

In search of a means of rapidly determining the cardiotoxic potential of anthracyclines, we have exposed suspensions of individual heart cells to ADR and have observed a dose-related termination of spontaneous beating. It is uncertain whether the mechanism of this acutely 'lethal' effect is related to the pathogenesis of chronic anthracycline cardiotoxicity in vivo. Nevertheless, the convenience and simplicity of this system might facilitate the initial toxic screening of analogs as well as further investigations into the mechanism of drug-induced lesions.

Materials and Methods

Adriamycin (NSC 123127), daunomycin (NSC 82151), desacetyladriamycin (NSC 268708), and 5-iminodaunomycin (NSC 254681) were obtained from the Division of Cancer Treatment, NCI.

Cardiac myocytes were prepared generally according to the method of Grosso et al. [6]. Male Sprague-Dawley rats (250–300 g) were injected IP with pentobarbital (60 mg/kg) and heparin (200 units/kg). Hearts were removed, immersed in ice-cold Ca^{2+}-free buffer (128 m*M* NaCl, 5.4 m*M* KCl, 8 m*M* Na_2HPO_4, 2 m*M* NaH_2PO_4, 15 m*M* glucose, pH 7.4), and perfused on an Ambec Extra-corporeal Perfusion Unit, Model 1000. Sixty milliliters of oxygenated Ca^{2+}-free buffer were pumped through the aorta at 37° C, 8–15 ml/min, to remove blood. Following preliminary digestion for 30 min with buffer containing 1 mg collagenase/ml (type II, Worthington), 1 mg hyaluronidase/ml (type I-S, Sigma), and 0.1 mg bovine serum albumin/ml (Fraction V, Miles or Sigma), hearts were removed and the ventricles minced for further digestion on a shaking incubator. Six consecutive 15-min digestions with 3 ml enzyme-buffer solution were each

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arrested with 10 ml ice-cold buffer; free cells were decanted, centrifuged at 30 *g*, and pooled. Further purification was obtained by two resuspension-settling steps and a gentle centrifugation through 3% buffered Ficoll [6]. All cell preparations were stored overnight at 4° C in Ca^{2+}-free buffer.

Viabilities of the preparation, as determined by either trypan blue exclusion or contraction characteristics, averaged approximately 70%–80% on the day of isolation. When newly isolated cells were resuspended in cold Tyrode's buffer (133.1 m*M* NaCl, 2.7 m*M* KCl, 1.9 m*M* $CaCl_2$, 11.9 m*M* $NaHCO_3$, 0.5 m*M* NaH_2PO_4, 6.0 m*M* glucose, pH 7.4), virtually all were in asytole 16 h later. However, when stored in the Ca^{2+}-free perfusion buffer at 4° C, 50%–60% of the cells retained their ability to contract on the following day. For studies of drug effects, cells were gently resuspended and diluted to $1-3 \times 10^6$ cells/ml. Control and experimental values were obtained by adding 4 µl cells to a hemacytometer with 8 µl double-strength Tyrode's buffer and 4 µl either distilled water or aqueous drug solution at 4 times the desired final concentration. Each slide was overlaid with a cover slip, and cells were observed at 100× magnification until all had ceased pulsing.

Results

Upon warming to room temperature (24°–26° C), individual myocytes began to contract spontaneously. For control cells kept in Ca^{2+}-free buffer, beating was fairly irregular and stopped completely by 20 min; however, when added to Ca^{2+}-containing Tyrode's buffer, cells beat regularly with individual rates varying from two to three contractions/s to one every 10–20 s. One by one, cells stopped contracting until all were acontractile by 73 ± 15 min (SD, $n = 36$). Myocytes evaluated in hemacytometers survived longer than those viewed on standard microscope slides, possibly owing to the greater volume of buffer.

Cells exposed to each of the four anthracyclines ceased contracting considerably earlier than control cells. Fig. 1 illustrates the appearance of the cells while under microscopic observation. During observation periods under 10 min, many cells suddenly rounded up in extreme, supercontracture, although a few of these continued to pulse for a short while. Cells contracting longer than 20 min seemed to gradually go into a progressive contracture from which they failed to recover completely; beating became slower and weaker. At the point when a cell ceased to contract, it could arrest in either a relaxed or a completely supercontracted state.

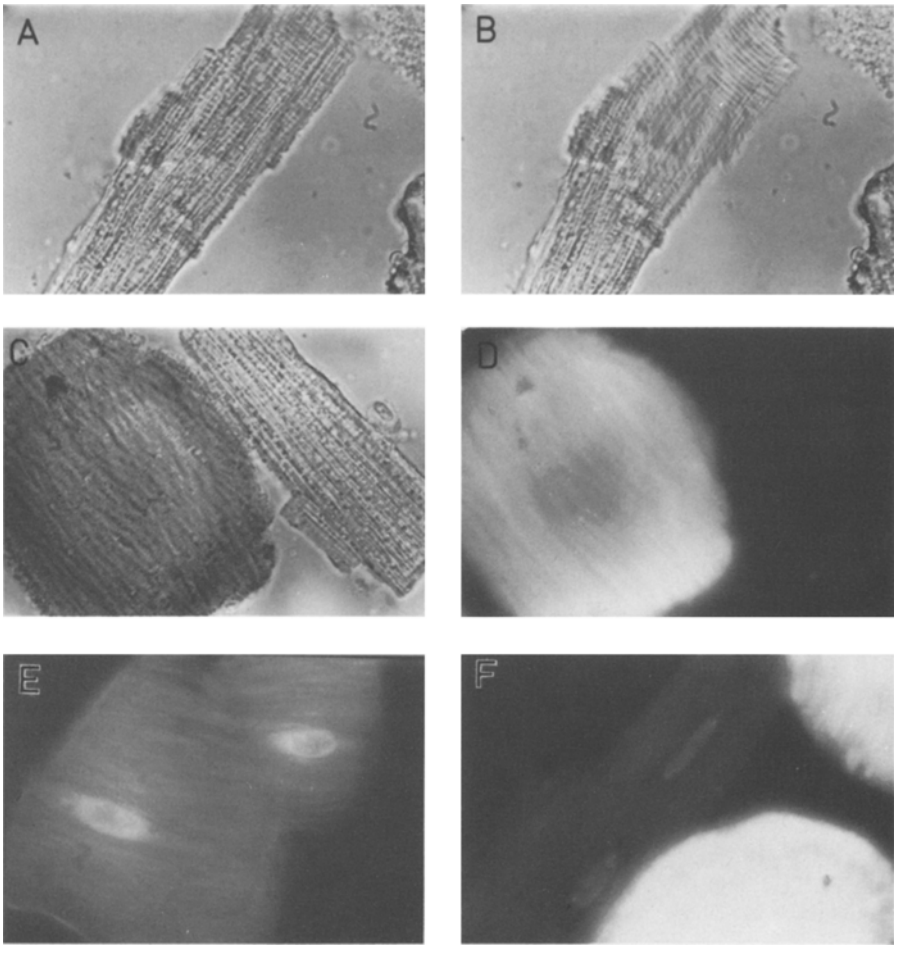

Fig. 1A–F. Photomicrographs of isolated adult rat heart myocytes: **A** Brightfield appearance of a single binucleated cell (440×); **B** Same cell as in **A,** contracting (440×); **C** Trypan blue staining of a non-contracting cell adjacent to a contracting cell which excludes the dye (420×); **D** Fluorescence view of C, in which contracting cell is non-fluorescent (420×); **E** Nuclear fluorescence of a cell exposed to adriamycin (1.7×10^{-4} *M*) which is in its last contraction at approximately 4 min (700×); **F** Fluorescence of a contracting cell exposed to adriamycin (1.7×10^{-4} *M*) for approximately 4 min, adjacent to two non-contracting cells fluorescent with both trypan blue and adriamycin (700×)

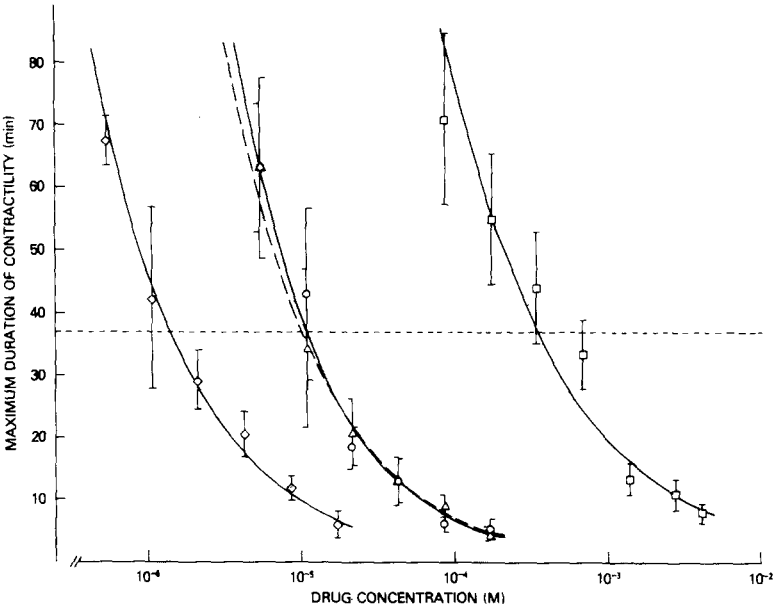

Fig. 2. Maximum beating times of isolated rat myocytes exposed to desacetyladriamycin (◇———◇), adriamycin (○———○), daunomycin (△———△), or 5-iminodaunomycin (□———□). Times were recorded when the last detectable contracting cell ceased to pulse (mean ± SD, $n = 6$). The mean maximum contraction duration of control cells is 73 min

Table 1. Effects of various agents on isolated myocyte preparation

	Concentration (10^{-4} *M*)	Average beating time[a] (min)
Agent		
Control	–	73 (± 15)
Antitumor agents		
Adriamycin	0.1	36
	1.0	7
Daunomycin	0.1	36
	1.0	7
Actinomycin D	1.0	65
5-Fluorouracil	10.0	> 73
Methotrexate	10.0	> 73
Mitomycin C	10.0	69–> 73
Mithramycin	10.0	> 73
Antibiotic		
Tetracycline	10.0	> 73
Cardioactive agents		
Isoproterenol	10.0	62
Ouabain	10.0	> 73
Prenylamine	1.0	54
	10.0	< 1
Other agents		
KCN	10.0	> 73
Dinitrophenol	1.0	55
	10.0	2
EDTA	10.0	70–> 73

[a] Duplicate determinations. If one value was less than 73 min, range is given

This pattern did not differ significantly from that of control cells – only the duration of beating was greatly affected. ADR at 100 μg/ml (1.7×10^{-4} *M*) caused a 92% inhibition of maximal beating times: while most cells ceased contracting by 3 min a few cells in each exposure consistently beat for 4–7 min. Dose-related inhibition of beating by each of the four anthracyclines showed a broad range of toxicities (10^{-6} *M* to 10^{-3} *M*, Fig. 2). By determining the concentration of each analog at the point its curve crosses the 37-min line (half the mean maximal contraction time of all controls), it is possible to assign a relative toxicity index to each drug (ID_{50}). By such a calculation, desacetyladriamycin is approximately 8 times as toxic as ADR or DAU (1.4×10^{-6} *M* to 1.1×10^{-5} *M*), whereas the latter are approximately 30 times more toxic than 5-iminodaunomycin (3.4×10^{-4} *M*).

To rule out the possibility of nonspecific cytotoxic effects, cells were exposed to other antitumor, cardiotoxic, or cytotoxic agents (Table 1). Only 2,4-dinitrophenol (DNP) and prenylamine (a Ca^{2+}-antagonist) significantly affected beating times, but at much higher concentrations than ADR or DAU (1 m*M*).

Discussion

Several reports have described heart cells prepared in this manner from both biochemical and morphological points of view [1, 3, 22], and do not suggest any apparent characteristic that would render this preparation unusually sensitive to toxic insult. We initially

questioned the reliability of determinations based upon the last contracting cell, for times at which 50% of cells ceased contracting might more accurately reflect behavior of a normal cell population; however, such discriminations cannot be made visually, given the numbers of cells and time limits involved. At less inhibitory doses of drug, endpoint determinations do seem to vary; but with multiple determinations repeated on several occasions, average beating times described by the curves in Fig. 2 have proven extremely reproducible.

Evaluating whether or not the concentrations of anthracyclines found to be toxic in this study are within ranges that might be encountered clinically, it can be calculated that the initial drug exposures in both situations are potentially quite similar. ADR at 60 mg/m^2 is commonly given as an IV push over 5 min or less. Assuming that a patient weighs 60 kg, and has a body surface area of 1.6 m^2, the amount of drug being administered is approximately 96 mg, or 19.2 mg/min. With a cardiac output of 100 ml/s or 6 litres/min and assuming no special accumulation or metabolism, a blood concentration of approximately 0.0032 mg/ml or 5.5×10^{-6} *M* would be reached at 1 min. Of the potential modes of metabolizing ADR, only the lungs would have an opportunity to lower the blood concentration of newly administered ADR before it first reached the left chambers of the heart. Even assuming significant pulmonary clearance, initial ADR levels in the coronary vasculature could range from 1 to 5×10^{-6} *M*. In other words, the concentrations of ADR found to be cardiotoxic in the isolated myocyte system are similar to those levels that might be attained clinically.

The mechanism of the effect of ADR and DAU upon beating times of these isolated myocytes is not known. It is possible that the observed effects are in some way related to the chronic cardiotoxicity for which the anthracyclines have become notorious. Interestingly, the heart cells used for these studies are remarkably resistant to other drugs such as actinomycin D and 5-fluorouracil, which are known to be extremely cytotoxic in tissue culture systems [21]. They commonly inhibit or suppress cell function in the micromolar range, but in this study they failed to affect beating times of isolated myocytes at millimolar concentrations. Thus the observed phenomenon does not seem to result from nonspecific cytotoxic effects. Moreover, as actinomycin D and cyanide are significantly more toxic in acute LD_{50} determinations in rodents than either ADR or DAU [7], this system does not merely reflect whole animal toxicities.

In Fig. 2, the parallel nature of the curves would suggest that the anthracyclines act upon isolated myocytes by a similar mechanism. Some documented actions of ADR and DAU, such as intercalating and damaging DNA or inhibiting mitochondrial respiration, do not appear solely responsible for the observed decreases in beating times. Actinomycin D induces similar frequencies of DNA breaks at approximately the same concentrations as ADR or DAU [19], and DNP is noted for its ability to uncouple oxidative phosphorylation; yet neither mimics the actions of the more potent anthracyclines on beating of isolated myocytes.

Interestingly, equimolar concentrations of ADR and DAU were indistinguishable in the isolated myocyte system (Fig. 2), yet they are quite different in whole animal toxicity (DAU has been reported to be 2–3 times as toxic as ADR in the mouse or the rat), in inhibition of mitotic activity of tissue cultures (DAU is often found to be 2–20 times as potent as ADR), and in their therapeutic doses as antitumor agents in several test systems (where there may be more than 2-fold differences) [7, 10, 14]. The in vivo differences in toxicity are most probably caused by pharmacokinetic/metabolic differences between these two drugs; the isolated myocytes would most probably demonstrate the inherent direct toxicity of the drugs, not that of their metabolites.

Three of the four anthracyclines studied in this report have also been evaluated in the rabbit histopathology screen [8], and have shown some apparent differences in relative toxicity from that found here. Desacetyladriamycin was relatively non-cardiotoxic in the rabbit and extremely toxic in the myocyte preparation, but the dosage used in the rabbit study was much less than that which would have been therapeutically comparable to ADR in the tumor screens. When doses equivalent to those required for reasonable chemotherapeutic responses in the tumor screen were used, rabbits died within 2–6 days, a period far too short to allow histopathologic evidence of the development of anthracycline cardiotoxicity. 5-Iminodaunomycin was found to be equally as cardiotoxic as ADR or DAU in the rabbit, but was considerably less toxic to the rat myocytes. These model discrepancies in predicting the cardiotoxic potential of desacetyladriamycin and 5-iminodaunomycin may be attributable to many factors, including species differences, disparate endpoints, drug metabolisms, acute vs chronic exposures, etc. This question cannot yet be resolved, for until the cardiotoxicities of several analogs are evaluated clinically, or until the mechanisms of action of anthracyclines in each system are known, we shall not know which of various models best predicts human toxicities.

Obviously, owing to the isolation and experimental treatment of these rat myocytes, one cannot

assume that their responses to anthracyclines accurately reflect those of human hearts. Yet it does appear that these drugs are inherently quite toxic to myocardial cells. Since it appears that the effects of ADR and DAU on myocyte contractions do not result from a generalized cytotoxicity, it may be possible to use the isolated myocyte system for preliminary in vitro evaluations of anthracycline cardiotoxicities. Unlike the in vivo rabbit model, which discriminates between analogs on a 0–3 histopathology scale, these myocytes seem to be extremely sensitive to differences between anthracycline analogs. In addition, many of the complicating factors of whole animal [25] or cultured heart [12, 20] studies are avoided, for there is virtually no contamination of the myocyte preparation by other cell types, results may be obtained quickly, there is no potential for metabolism of the drug by other tissues, and the observed effects cannot be secondary to, or masked by, other systemic toxicities. Cells are uniformly exposed to known concentrations of drugs, and experimental conditions can be easily controlled and modified. Thus, because of the apparently specific effects of anthracyclines, isolated rat myocytes might prove useful in investigating the actual mechanism of anthracycline cardiac toxicity, which is not yet understood although numerous reports have suggested involvement of suppressed DNA synthesis [5, 18], impaired mitochondrial respiration [4, 11], lipid peroxidation [16], or reduced cellular Ca^{2+} flux [17, 23].

Finally, the absence of other cell types in this preparation and the elimination of whole animal physiological barriers may make this system ideal for studying the specific mechanisms by which a variety of toxic agents affect the heart.

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