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The Effects of Adriamycin on Intracellular Calcium Concentrations of L1210 Murine Leukemia Cells

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Abstract—*Changes in the concentration of intracellular Ca^{2+} may be an important component of the mechanism of adriamycin toxicity to tumor cells. Adriamycin interacts with the plasma membrane, a phenomenon which may lead to a direct modulation of Ca^{2+} transport proteins or, since the drug is a quinone, may lead to indirect changes in Ca^{2+} homeostasis induced by oxidative stress to the cell. The calcium content of L1210 murine leukemia cells treated with adriamycin for up to 6 hr was estimated using the cell-impermeant dye arsenazo-III. Pools of intracellular Ca^{2+} were released to the extracellular compartment, where they reacted with the dye by sequential treatment of the cells with m-fluorocarbonylcyanidediphenylhydrazone (FCCP) and the Ca^{2+}-ionophore A23187. Pretreatment of L1210 cells with ruthenium red (5 μM) selectively decreased the FCCP-releasable Ca^{2+} pool, which suggested it was mitochondrial in origin. Continuous exposure of L1210 murine leukemia cells* in vitro *to 5 or 10 μM adriamycin for 2 hr did not produce any change in the intracellular concentration of releasable Ca^{2+}; at 4 hr, however, the total releasable pool of Ca^{2+} rose by 29% and 46% for 5 and 10 μM adriamycin respectively. This increase was seen predominantly in the mitochondrial pool. Exposure of L1210 cells to the quinone, menadione, also increased the releasable pools of cellular Ca^{2+} but like adriamycin, only after an incubation period of 4 hr. These results contrasted with a rapid decrease in mitochondrial Ca^{2+} concentration produced by a short (5 min) exposure to 500 μM t-butyl-hydroperoxide, a generator of free radicals. After treatment with 8 mM lidocaine, a membrane fluidizing agent, there was a rapid fall in extramitochondrial Ca^{2+}. These findings suggest that (a) changes in L1210 Ca^{2+} homeostasis induced by adriamycin and menadione are late, and possibly common, events of quinone toxicity to L1210 cells, (b) adriamycin does not have an immediate effect on Ca^{2+} ion transport produced by the direct interaction of the antibiotic with the plasma membrane, and (c) oxidative stress induced by redox-active quinones may not be important for the induction of toxicity in neoplastic cells.*

INTRODUCTION

THE MECHANISM by which adriamycin (ADR) exerts its toxic action on normal and neoplastic cells is as yet undefined. This amphipathic drug may cause irreversible damage to DNA and/or cellular membranes by direct interaction as an intercalating agent [1, 2], or by indirect effects via the formation of oxygen free-radicals [3–6]. Interactions with membranes could result in gross changes in cellular Ca^{2+} homeostasis, which may contribute to cytotoxicity if concentrations of free cytoplasmic Ca^{2+} were changed beyond tolerable thresholds [7].

Direct, early (< 2 hr) effects of ADR on membranes have been observed as profound increases in plasma membrane fluidity of mouse sarcoma 180 cells [8]. Such changes could modulate the activities of key membrane-bound enzymes, such as the calmodulin-dependent Ca^{2+} ATPase and the Na^+–Ca^{2+} antiporter. Inhibition of the Na^+–Ca^{2+} antiporter of heart cells has been demonstrated *in vitro* at ADR concentrations of 10 μM and above [9]; this may lead to a rise in cytoplasmic Ca^{2+} and an overloading of the buffering capacity of cardiac mitochondria for Ca^{2+}. If this also occurred in a tumor cell, its ability to regulate cytoplasmic Ca^{2+} concentration would be compromised.

Indirect effects of ADR on critical cellular macro-

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Abbreviations—ADR, Adriamycin (doxorubicin); EGTA, [ethylene-bis(oxyethylenenitrile)]tetraacetic acid; FCCP, *m*-fluorocarbonylcyanidediphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HS, horse serum; t-BuOOH, t-butylhydroperoxide; DMSO, dimethylsulfoxide.

molecules may also occur through redox-cycling of the quinone portion of the molecule, a process which produces potentially lethal oxygen free-radicals. In hepatocytes, the redox-cycling of the quinone menadione catalyzed by NADPH-cytochrome *c* reductase, brings about a rapid (< 20 min) fall in mitochondrial and extra-mitochondrial Ca^{2+} concentrations [10–13]. This inability of hepatocytes to sequester Ca^{2+} was secondary to a fall in reduced glutathione, and is considered to be responsible for a profound disturbance of the cytoskeleton which ultimately leads to a loss in cellular integrity [12–14]. These toxic effects may be ameliorated in intact cells by the enzyme DT-diaphorase which catalyzes the two electron reduction of menadione to a non-toxic hydroquinone [13–15]. It is possible that an analogous redox-cycling of ADR in tumor cells may occur and bring about similar changes in the ability of tumor cell mitochondria to sequester Ca^{2+}.

For these reasons, we have studied the effects of ADR on the Ca^{2+} homeostasis of murine leukemia cells incubated *in vitro* with concentrations of ADR which are at the upper limits of its pharmacologically relevant cytotoxic range. The profile of ADR-induced changes in L1210 cell Ca^{2+} homeostasis was compared with that induced by agents known to produce oxidative stress to cells either directly (t-BuOOH) or through redox-cycling (menadione) and with that caused by an agent known to change intracellular Ca^{2+} concentrations subsequent to a change in membrane fluidity (lidocaine).

MATERIALS AND METHODS

Chemicals were obtained from the following sources: ADR (National Cancer Inst., Bethesda, MD), menadione and t-BuOOH (Aldrich Chemical Co., Milwaukee, WI), HEPES buffer, FCCP, lidocaine and arsenazo-III (Sigma Chemical Co., St. Louis, MO), A23187 (Calbiochem-Behring, San Diego, CA), Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (10×), Fischer's medium (Grand Island Biological Co., Grand Island, NY) and horse serum (Flow Laboratories, McLean, VA). Gases were purchased from Presto Welding Service Centers (North Haven, CT).

For drug treatment prior to estimation of cellular Ca^{2+}, exponentially growing L1210 cells maintained in Fischer's medium, containing 10% HS, with or without drugs were incubated in this growth medium at a level of $2–5 \times 10^5$ cells/ml at 37° C with continuous shaking in an atmosphere of 95% air/5% CO_2. Experiments were started by the addition of vehicle or drug; ADR, ruthenium red and lidocaine were dissolved in Ca^{2+}-free buffer, and menadione was dissolved in DMSO (added to 0.5%, v/v). At appropriate times, 1×10^7 cells were harvested, washed with ice-cold, Ca^{2+}-free HEPES–Hank's buffer (prepared as outlined below) and resuspended in 1.1 ml of this Ca^{2+}-free buffer at room temperature. One ml of the cellular suspension in Ca^{2+}-free buffer was placed in a cuvette at room temperature and the remainder of the cell suspension was used to measure cell number using a Coulter ZB_1 counter. After the sequential addition of FCCP (15 μM) and A23187 (10 μM) in a final concentration of 1% DMSO the releasable Ca^{2+} concentration excreted into the medium was determined by monitoring the change in absorbance of the externally located dye arsenazo-III (24 μM). Changes in absorbance were measured using an Aminco-Chance spectrophotometer in the dual beam mode at 660–700 nm. This method was similar to that described previously for use with hepatocytes [11, 13, 14, 16] and essentially L1210 cells behaved in the same way. The assay in Ca^{2+}-free medium was completed within 15 min, during which time no Ca^{2+} loss to the medium was detected from cells which were not treated with either FCCP or A23187. Cell viability was assessed by exclusion of 0.1% trypan blue in saline.

The extent of the absorbance changes produced by FCCP and A23187 was dependent on cell number. Intra-assay reproducibility was good (± 10% S.D.) but inter-assay variability was high, being influenced by the density at which the cells were harvested. The percentage S.D. was ± 20% for the presumed mitochondrial pool, ± 21% for the extramitochondrial pool and ± 18% for total cell mobilizable Ca^{2+}. In the experiments reported, variability was minimized by stringent control of the seeding and harvesting densities of the cellular preparations.

The Ca^{2+}-free buffer was prepared by dissolving 20 mM HEPES in Hank's balanced salt solution without Ca^{2+} and Mg^{2+}, and was brought to pH 7.4 with NaOH. The buffer was made Ca^{2+}-free by monitoring the Ca^{2+} content in a 1 ml aliquot of buffer with arsenazo-III (24 μM), and titrating with 5 μM EGTA until the absorbance reached a minimum of approx. 0.05 absorbance units.

Single additions of 5 μM $CaCl_2$ were then made to calculate the stepwise change in absorbance over the range of 0–50 μM Ca^{2+}; absorbance was linear up to 40 μM Ca^{2+}. Back titration with EGTA allowed an estimate to be made of the Ca^{2+} content of the HEPES–Hank's buffer, and sufficient EGTA (approx. 23 μM) was added to the buffer to bring this value to zero. All solutions were prepared in deionized distilled water and stored in plastic containers.

Enzyme activities were assayed in whole cell sonicates prepared by washing cells with cold phosphate buffered saline and resuspending in 6 vol. of deionized distilled water. After incubation on ice

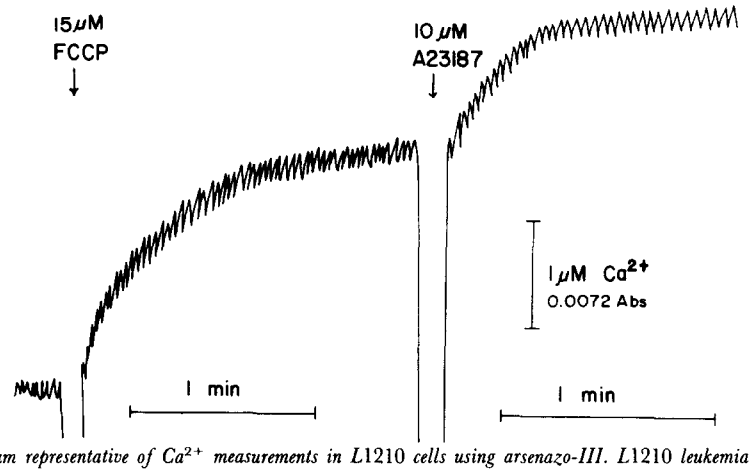

Fig. 1. Spectrum representative of Ca^{2+} measurements in L1210 cells using arsenazo-III. L1210 leukemia cells (10^7/ml) in 20 mM HEPES–Hank's Ca^{2+}–Mg^2-free buffer (pH 7.4) containing 24 μM arsenazo-III were monitored at 660–700 nm for changes in absorbance brought about by the sequential addition of 15 μM FCCP and 10 μM A23187 at room temperature.

for 10 min, 1.8% NaCl was added to make the suspension isotonic, and this was followed by sonication of cells 3 times for 6 sec each. Enzyme activities were then measured by conventional techniques as follows: NADPH-cytochrome *c* reductase activity was determined at 30° C by monitoring the rate of reduction of cytochrome *c* at 550 nm [17] using an extinction coefficient of 27.7 mM^{-1} cm^{-1}. DT-diaphorase activity was assayed at 30° C by reduction of dichlorophenolindophenol (600 nm, ϵ=21 mM^{-1} cm^{-1}) in the presence and absence of 100 μM dicoumarol [18].

RESULTS

Arsenazo-III was used to measure intracellular pools of Ca^{2+} in L1210 cells by methodology developed for hepatocytes [11, 13, 14, 16]. Figure 1 shows that the addition of FCCP, an uncoupler of the mitochondrial proton gradient, caused the release of calcium from the cells into the extracellular Ca^{2+}-free HEPES–Hank's buffer where it reacted with the impermeant arsenazo-III, and produced a time-dependent change in absorbance at 660 nm. It is presumed that as the cytoplasmic Ca^{2+} concentration was elevated by the release of mitochondrial Ca^{2+}, the cell restored the cytoplasmic Ca^{2+} concentration to the norm by pumping excess calcium out of the cell via the Ca^{2+}-pump or by Na^+–Ca^{2+} exchange. Figure 1 also shows the increase in the Ca^{2+}–arsenazo-III signal occurring when the calcium ionophore A23187 was added subsequent to FCCP, allowing the Ca^{2+} remaining in the cell to become equilibrated passively across the plasma membrane. The FCCP-induced signal is taken to be a measure of the mobilizable mitochondrial Ca^{2+} concentration, and the A23187-induced signal, a measure of the mobilizable cytoplasmic or extramitochondrial Ca^{2+} concentration [11]. Addition of 15 μM A23187 only, effectively released the total Ca^{2+} content of the cell (data not shown), a finding analogous to that reported for heptocytes [11]. No change occurred in the absorbance of arsenazo-III over the time period of the assay (approx. 10 min) upon the addition of vehicle only.

The assay procedure employed was further validated, in terms of the assumptions made above regarding the separate pools of intracellular calcium, by incubating L1210 cells with ruthenium red, to specifically decrease mitochondrial Ca^{2+} content by blockade of the mitochondrial Ca^{2+} uniporter [19]. Table 1 shows that incubation of L1210 cells for 5 min with 5 μM ruthenium red decreased the mitochondrial Ca^{2+} content in a manner similar to that reported in isolated hepatocytes [11]. As the time of incubation with ruthenium red was increased to 10 min, a loss of extramitochondrial Ca^{2+} was also observed, which coincided with a decrease in cell viability from 95 to 81% (data not shown).

The effects of incubation of L1210 cells with ADR on the releasable Ca^{2+} pools of L1210 cells are shown in Fig. 2. After 2 hr exposure to 5 or 10 μM ADR there was no significant change in the total (FCCP + A23187), mitochondrial (FCCP) or extramitochondrial (A23187) pools of releasable Ca^{2+} in L1210 cells. At 4 hr, total cellular Ca^{2+} concentration increased at both concentrations of ADR. Five μM ADR increased both mitochondrial and extramitochondrial Ca^{2+}. Ten μM ADR had a minimal effect on extramitochondrial Ca^{2+} but greatly increased mitochondrial Ca^{2+}. Why 5 μM but not 10 μM should increase extramitochondrial Ca^{2+} is not clear. However, at 6 hr the increase in cellular Ca^{2+}, which was maintained, by both 5 and 10 μM ADR, was almost entirely due to increases in the mitochondrial pool, and clearly it is this pool which ADR most affected. During the 6 hr period of exposure to the drug, exclusion of trypan blue remained > 90%, suggesting that the plasma mem-

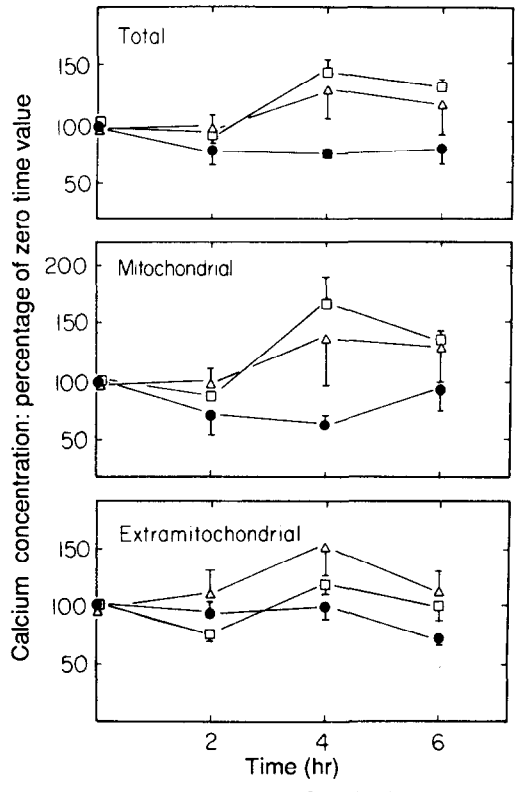

Fig. 2. Changes in releasable cellular Ca^{2+} induced by adriamycin. Two to 5 × 10^5 L1210 cells per ml were incubated in Fischer's medium containing 10% HS with 5 or 10 μM adriamycin at 37° C for the time periods shown. The changes in releasable Ca^{2+} were compared to the values obtained from cells immediately after addition of drugs or vehicle. The intracellular Ca^{2+} concentration was measured with arsenazo-III by the assay described in the Materials and Methods. Initial values for mitochondrial, extramitochondrial, and total releasable Ca^{2+} were 160, 120 and 280 nM/10^6 cells, respectively. Data points represent the mean ± S.E. of 4 determinations. ●, vehicle control; △, 5 μM adriamycin; □, 10 μM adriamycin.

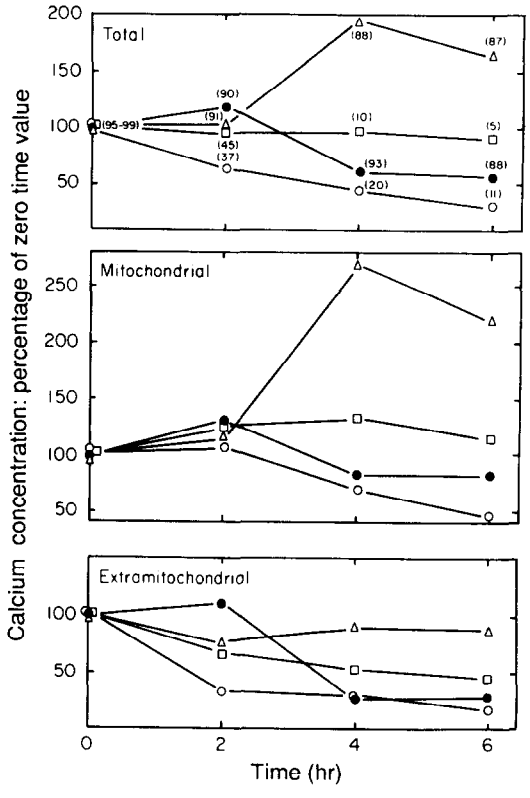

Fig. 3. Changes in releasable cellular Ca^{2+} induced by menadione. Two to 5 × 10^5 L1210 cells per ml were incubated in Fischer's medium containing 10% HS with 5, 50 or 200 μM menadione at 37° C for the time periods shown. Numbers in parentheses show the % cell viability as measured by the exclusion of trypan blue. The changes in releasable Ca^{2+} were compared to zero time values and were measured as described in the Materials and Methods. Initial values for mitochondrial, extramitochondrial and total releasable Ca^{2+} were 105, 95 and 200 nm/10^6 cells. Data points are from representative experiment. ●, vehicle control; △, 5 μM menadione; □, 50 μM menadione; ○, 200 μM menadione.

brane was intact at this time, although > 99.99% of the cells were destined to die when cellular viability was assessed by cloning, as determined in this study (data not shown) and by others [20, 21].

Figure 3 shows the effect of menadione on the mobilizable Ca^{2+} pools of L1210 cells. At 5 μM menadione, where short-term viability was maintained, little effect on Ca^{2+} homeostasis was observed until after 4 hr of incubation, whereupon total releasable Ca^{2+} doubled. This increase in Ca^{2+} was largely accounted for by an increase in the mitochondrial pool, in a manner analogous to that produced by ADR. Exposure of L1210 cells to 50 μM menadione resulted in no apparent changes in intracellular Ca^{2+}. Interpretation of these data, however, is complicated by the finding that cell viability decreased during the course of the experiments with this higher concentration of menadione. Since determination of cell viability by trypan blue exclusion is a reasonable measure of plasma membrane integrity, the lack of change in cellular Ca^{2+} at high concentrations of menadione may be the result of leakage of Ca^{2+} from non-viable cells into the Ca^{2+}-free buffer used to wash the cells immediately prior to assay. Correction of the data to correspond to the number of viable cells indicates that intracellular Ca^{2+} increased in cells with intact membranes.

Exposure of L1210 cells to the radical generating compound t-BuOOH rapidly lowered the intracellular Ca^{2+} concentration. This resulted from a parallel decrease in both mitochondrial and extramitochondrial Ca^{2+} (Table 1). During the 5 min exposure of cells to t-BuOOH, the intregrity of the plasma membrane remained intact, as measured by trypan blue exclusion. Measurements involving longer periods of exposure to t-BuOOH were not performed since viability was reduced and it appeared that the plasma membrane did not remain intact under these conditions.

Local anesthetics increase the fluidity of cell plasma membranes [22], as does ADR [8], producing changes in membrane function. Concentrations of lidocaine of up to 10 mM had no effect on the uptake of trypan blue (Table 1) or on clonogenicity (data not shown) over a 1 hr period

Table 1. Changes in free intracellular Ca^{2+} in L1210 cells

Treatment*	Mitochondrial	Extramitochondrial % of vehicle control†	Total	Viability
t-BuOOH	64 ± 7‡	58 ± 7‡	62 ± 6‡	90
Lidocaine	90 ± 6§	67 ± 10§	79 ± 8‡	94
Ruthenium red	48 ± 16§	73 ± 2‖	57 ± 9§	93

*L1210 cells were treated for 5 min with 500 μM t-BuOOH, for 1 hr with 10 mM lidocaine, or for 5 min with 5 μM ruthenium red.

†Experiments were performed as detailed in Materials and Methods. Each value represents the results of 3–4 determinations ± S.E. Initial values for mitochondrial, extramitochondrial and total Ca^{2+} concentrations were 156, 116, and 272 nM/10^6 cells, respectively, for t-BuOOH treated cells and 171, 121, 292 nM/10^6 cells, respectively, for ruthenium red and 219, 196, 415 nM/10^6 cells, respectively for lidocaine treated cells. Significance levels, by Students' *t*-test:

‡ $P \geq 0.01$; § $P < 0.001$; ‖ $P < 0.005$.

Table 2. Oxidoreductase activities

	NADPH-cytochrome *c* reductase*	DT-Diaphorase†	Ratio
	nmol/min/mg cellular protein		
L1210 leukemia	11.8	<10.0	>0.8
Rat liver‡	45	400	8.9

*Activity was determined by the reduction of cytochrome *c* at 30° C as described in the Materials and Methods section.

†Activity was determined as described in the Materials and Methods section by monitoring the reduction of dichlorophenolindophenol at 30° C.

‡Calculated from References 22 and 23.

of exposure. A 1 hr incubation with lidocaine caused a decrease in total mobilizable Ca^{2+} primarily brought about by a reduction in the extramitochondrial Ca^{2+} pool (Table 1).

Activities representative of NADPH-cytochrome *c* reductase and DT-diaphorase, which are involved in the bioactivation of quinones, and may indirectly modulate Ca^{2+} homeostasis [13, 15], were measured in whole cell sonicates of L1210 murine leukemia cells (Table 2). NADPH-cytochrome *c* reductase activity was present in L1210 cells, but activity was lower than that found in rat liver [23]. DT-diaphorase activity was virtually absent from the leukemic cells in contrast to the high activity present in liver [24].

DISCUSSION

ADR may change cellular Ca^{2+} homeostasis directly by interaction with membranes [2, 8–9, 25–27] or indirectly by oxidative stress produced by redox-cycling [3, 6, 15]. Both interactions could lead to profound changes in intracellular Ca^{2+} pools, an event which could be cytotoxic. Treatment of L1210 cells with pharmacologically relevant concentrations of ADR (5 and 10 μM) did not change the Ca^{2+} concentration in either the mitochondrial or extramitocondrial pools until after 2 hr of incubation (Fig. 2). At later times, an increase occurred in the concentration of the mitochondrial and extramitochondrial Ca^{2+} pools. This effect was compared to that produced by menadione and t-BuOOH which have been reported to cause oxygen-dependent damage to cells, and have been extensively studied using isolated hepatocytes [10–15], and to that produced by lidocaine, an agent which, like adriamycin, fluidizes plasma membranes [8, 22].

The quinones, menadione and ADR, are capable of generating a semiquinone radical, and potentially cytotoxic oxygen radicals, through reduction by flavoproteins. In isolated hepatocytes a 20 min exposure to 50 μM menadione reduced total releasable Ca^{2+} by 50% with no loss of cell viability; this change was due to a decrease in all intracellular Ca^{2+} pools [13]. In L1210 cells, 50 μM menadione decreased the cell viability to an extent that made interpretation of changes in intracellular Ca^{2+} pools difficult. At 5 μM menadione, however, L1210 cells remained viable and Ca^{2+} levels increased, with a marked accumulation of mitochondrial Ca^{2+} occurring after 4 hr of exposure; no increase in intracellular Ca^{2+} was observed at 2 hr, a result similar to that found for ADR (Fig. 3). These findings suggest that menadione may be toxic to leukemic cells by a mechanism different from that reported for hepatocytes, and by implication, a

similar situation might well exist for ADR. It is conceivable that in leukemic cells, unlike in hepatocytes, oxidative stress caused by redox-cycling may not be the primary cause of cytotoxicity. This latter possibility is supported by the findings obtained with L1210 cells exposed to t-BuOOH (Table 1). In this case, L1210 leukemia cells behaved like hepatocytes with respect to their Ca^{2+} homeostasis after being challenged with this oxygen free-radical generator. The production of oxygen free-radicals has been shown to bring about a fall in cellular non-protein thiols such as glutathione [10, 13]. This decrease results in a lower capacity of mitochondria to sequester Ca^{2+} from extramitochondrial pools, and a rapid fall in mitochondrial Ca^{2+} ensues. Such a phenomenon appears to occur in L1210 cells treated with 500 μM t-BuOOH for 5 min. These results raise the question of whether oxygen radical damage is involved in the toxicity of ADR to tumor cells since a fall in mitochondrial Ca^{2+} was not observed. We have demonstrated (Table 2) that L1210 leukemia cells contain NADPH-cytochrome *c* reductase activity, an enzyme capable of reducing ADR and menadione with subsequent reduction of oxygen to generate cytotoxic oxygen radicals [3–6]. Therefore L1210 cells are presumably able to reduce ADR and menadione with subsequent generation of cytotoxic oxygen radicals. However, ADR and menadione at concentrations below 50 μM did not cause a decrease in Ca^{2+} levels in L1210 cells, in contrast to the results obtained with t-BuOOH. Although the contribution of oxygen free-radical damage to the cytotoxicity of ADR to leukemic cells remains unresolved, our findings indirectly support the hypothesis that oxygen free-radicals play little or no major role in the toxicity of ADR to these cells, particularly in light of evidence that the cytotoxicity of ADR is not dependent upon oxygen [28, 29].

With regard to possible direct actions of ADR on plasma membrane function, ADR causes the accumulation of mitochondrial Ca^{2+} in damaged cardiac tissue [30, 31], but only at concentrations above the upper limits of measurable toxicity to L1210 cells. Katoh *et al.* [32] have shown that 10 μM ADR inhibited calmodulin sensitive kinase activity in heart, and Caroni *et al.* [9] have suggested that 10 μM ADR blocks Na^+–Ca^{2+} exchange. Both of these effects would normally demand that the cell reduce an overloading of the normally low cytoplasmic Ca^{2+} concentration by sequestering it into mitochondria and possibly other intracellular storage sites. This phenomenon may explain our finding that long-term exposure to ADR produced an elevated mitochondrial Ca^{2+} concentration in L1210 cells; such an effect would occur if both the Na^+–Ca^{2+} exchanger and the calmodulin-sensitive Ca^{2+}-pump in the plasma membrane were damaged. However, it is clear that these alterations are not early events in the action of ADR, even at highly cytotoxic concentrations. In addition, the fact that FCCP can release elevated mitochondrial Ca^{2+} pools to the exterior of the cell, a process supposedly dependent upon the action of the Ca^{2+} pump and/or the Na^+–Ca^{2+} exchanger, even after 6 hr of exposure of L1210 cells to ADR, suggests that pump-mediated exchange of ions across the plasma membrane is still operative. Nevertheless, the fact that L1210 cells treated with adriamycin begin to accumulate calcium after 2 hr may be an important factor that contributes to the ultimate death of these cells, even if it is not the primary, causative lesion.

We have previously reported data which show that the Ca^{2+} pump of human red blood cells is unaffected by ADR at a level as high as 100 μM [33], and studies of the effects of ADR on L1210 cell membrane potential and rubidium ion flux similarly show it to have no early effect on ionic homeostasis modulated by the plasma membrane [34]. In addition, the local anesthetic lidocaine, a plasma membrane fluidizing agent like ADR, affected only extramitochondrial Ca^{2+} (Table 1). Thus, the present results suggest that although ADR is capable of interacting with cell membranes to rapidly change parameters such as fluidity [8], the function of plasma membrane associated proteins concerned with Ca^{2+} homeostasis appears to be unchanged.

In conclusion, L1210 cells treated with ADR show no early changes in Ca^{2+} homeostasis. Later changes, particularly an increase in mitochondrial Ca^{2+}, are not compatible with a mechanism of oxygen free-radical induced damage, raising doubts about the contribution of such damage to ADR cytotoxicity. The absence of early changes that would reflect an altered capability of the plasma membrane to expel Ca^{2+} suggests that ADR has no readily detectable, early effect on Ca^{2+} homeostasis and provides further evidence that the antibiotic does not rapidly alter ion transport mediated by the plasma membrane. This observation is compatible with a previous report that the drug had no early effects on rubidium transport or on the membrane potential of L1210 cells [34]. Further investigations to ascertain the precise, early effects induced by the interaction of ADR with membranes are underway, and are presently focussed on drug–cytoskeletal interactions [35].

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