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TOXIC EFFECTS OF DAUNORUBICIN ON ISOLATED AND CULTURED HEART CELLS FROM NEONATAL RATS

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Abstract—Various aspects of the cardiotoxicity of the anthracycline derivative and antineoplastic drug daunorubicin were investigated using isolated and cultured cells from neonatal rat hearts as a model system. Treatment of the cells with concentrations of daunorubicin of the same order of magnitude as those used in chemotherapy was accompanied by marked toxic effects, e.g. a decreased or abolished contraction, and release of lactate dehydrogenase, pyruvate and oxidized glutathione to the medium. A decreased frequency of contraction appeared to be the most sensitive probe of daunorubicin toxicity, followed by release of pyruvate and oxidized glutathione/lactate dehydrogenase. Daunorubicin and/or its metabolites also bound to cellular protein and DNA. Exposure to daunorubicin was shown to be accompanied by a rapid induction of primarily DT-diaphorase and a slower induction of glutathione transferase. The latter observations are interpreted to indicate a protective role of quinone- and peroxide-metabolizing enzymes, respectively, and support the hypothesis that daunorubicin toxicity involves generation of free radical derivatives, which initiate lipid peroxidation. This conclusion is further substantiated by the demonstration that addition of daunorubicin leads to an increased oxygen consumption.

Isolated contracting heart cells from neonatal rats have previously been employed as a model system in studies of the mechanism of action of various drugs, including anticancer drugs of the anthracycline type, in heart tissue [1–6]. In the present investigation this model system was used in order to further characterize the cardiotoxic effects of the anthracycline daunorubicin (DRB). DRB is a drug widely used in the treatment of different malignancies, especially leukemias [7]. However, the clinical use of DRB is limited by various toxic, especially cardiotoxic, side effects [8–10]. DRB is also mutagenic and carcinogenic in man [11].

Although it is generally accepted that the antitumour action of DRB to some extent is due to its capacity to intercalate between the DNA strands, this mechanism is not sufficient to explain the cardiotoxicity of DRB. The current leading hypothesis involves the formation of a semiquinone free radical by enzymatic single-electron reduction [12]. These semiquinones can then initiate chain reactions involving oxygen free radicals which in turn result in cell injury and cell death. Other effects of DRB involve a decrease in heart metabolism [4, 13], inhibition of the respiratory chain [14] and alterations in the transport of various ions [15]. In addition, it has been shown that drugs of the anthracycline type may bind to DNA [16, 17] and cause a decrease in myocardial protein and RNA content [18, 19]. Binding to DNA has also been proposed to involve reactive species produced after the reduction cleavage of the sugar moiety [20].

Previous work in our laboratory has shown that DT-diaphorase (a two-electron quinone reductase) and glutathione transferase are induced 2- to 4-fold in isolated neonatal heart cells in culture following exposure to DRB [21]. Moreover, pretreatment with dicoumarol, a potent inhibitor of DT-diaphorase [22], increased the sensitivity of the cells to DRB, indicating a protective role of the enzyme. These results support the hypothesis that DT-diaphorase competes with one-electron reductases for DRB and that the hydroquinone products are relatively harmless [23].

In the present report, the toxic effects of DRB on cultured neonatal heart cells have been further investigated. Covalent binding of DRB to cellular protein and DNA, DRB-dependent changes in release of lactate dehydrogenase (LDH) and cellular components, beating frequency oxygen consumption and activities of DT-diaphorase and glutathione transferase were determined as a function of time of exposure and concentration of DRB. The results support the contention that free radical mechanisms are important in DRB toxicity.

MATERIALS AND METHODS

Isolation of neonatal heart cells. The method of isolating heart cells from neonatal rats and the subsequent establishment of primary cultures of these cells were carried out as described previously [24]. Growth medium was changed daily and drug treatment was initiated 3 days after plating the cells; at this time the cells were beating at a constant frequency of about 150 contractions/min. In all cases the Petri dishes (6 cm in diameter) contained $5 \cdot 10^6$ cells. Labelled DRB was added to the cells as $3 \cdot 10^5$ and 10^6 dpm of (^{3}H)DRB when covalent binding to protein and DNA, respectively, was measured.

DRB uptake and release and covalent binding to protein. The growth medium from Petri dishes treated with (^{3}H)-DRB was discarded and the cells were washed with 20 mM Tris–HCl (pH 7.3), scraped off and collected in 1 ml per dish of the same buffer. The radioactivity of the cells was taken as a measure of total DRB bound by the cells. Release of DRB from the cells was measured after pretreatment with (^{3}H)-DRB for 1 day, after which the cells were washed with fresh medium. The subsequent appearance of radioactivity in the medium was used as a measure of DRB released. In order to estimate covalent binding of DRB to protein, the cells were sonicated in a batch sonicator twice for 30 sec and centrifuged at 10,000 *g* for 5 min in a table centrifuge. 50 μl of the supernatant was then applied on a cellulose filter, after which the filter was washed extensively with various organic solvents as described [25]. After washing the radioactivity bound to the protein on the filter was measured in a scintillation counter and expressed as cpm/50 μl supernatant.

Covalent binding of DRB to DNA. After treatment of the cells with (^{3}H)-DRB for the time indicated, medium was discarded and the cells were collected after scraping in 2 ml per dish of buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 10 mM NaCl and 0.5% SDS. The cells were then sonicated twice for 30 sec in a batch sonicator and DNA was isolated as described [26]. DNA isolated by this method had a 260/280 nm absorption ratio of approximately 1.95 (cf. [27]), and the specific radioactivity content determined by scintillation counting, was expressed as cpm/μg DNA.

Beating frequency and amplitude. Beating frequencies in normal growth medium were estimated visually by a microscope by counting the number of beats in 5 different areas (4 in the periphery, 1 in the centre) of each of at least 3 dishes treated with the drug concentration indicated. Each point represents an average of these determinations. Alternatively, amplitude and frequency of the contractions were continuously and simultaneously measured by using a highly sensitive monitor previously designed in this laboratory [24].

Release of LDH. The activity of lactate dehydrogenase was estimated spectrophotometrically at 340 nm in the presence of 2 mM pyruvate and 200 μM NADH. Medium was 50 mM Tris–HCl (pH 7.3) and temperature was 30°.

Release of pyruvate and GSSG. Extracellular pyruvate was estimated by following NADH oxidation spectrophotometrically at 340 nm. The reaction was carried out with a medium containing Tris–HCl (pH 7.3), 200 μM NADH, 10 μg LDH and 200 μl sample in a total volume of 3 ml. Temperature was 30°.

Extracellular oxidized glutathione (GSSG) was estimated by following NADPH oxidation spectrophotometrically at 340 nm. The reaction was carried out in a medium containing 0.1 M sodium phosphate (pH 7.6), 100 μM NADPH, 0.5 mM EDTA, 0.1 mg glutathione reductase and 100 μl sample, in a total volume of 1 ml. The temperature was 30°.

Induction of glutathione transferase and DT-diaphorase. Activities of glutathione transferase and DT-diaphorase were determined directly using cell homogenates as described previously [21]. Glutathione transferase was assayed with chlorodinitrobenzene [28], whereas DT-diaphorase was assayed with cytochrome *c* [29].

Oxygen consumption. A highly sensitive method for measuring oxygen consumption of cultured cells was developed by Petterson and Walum [30]. The method uses a continuous flow system in which the cells are exposed to a growth medium containing the various additions in minimal essential medium. The oxygen consumption of the cells is then estimated by 2 Clark electrodes which monitor the oxygen concentration of the medium before and after it has passed the cells. Control values were about 40 μl oxygen consumed/mg protein/hr.

Chemicals. DRB (Cerubidin) was obtained from Huddinge University Hospital (Huddinge, Sweden). (^{3}H)-DRB (4.3 Ci/mmole) was purchased from New England Nuclear (Boston, MA). Growth medium and antibiotics were obtained from Gibco (Grand Island, NY), LDH (canine muscle) and glutathione reductase (yeast) and all other biochemicals from Sigma Chem. Co. (St. Louis, MO).

RESULTS

As shown in Fig. 1, labelled DRB accumulated in cultured heart cells and was extensively extracted from the culture medium; 24 hr after the addition of DRB the concentration of DRB in the medium had decreased to less than 5% (not shown). Cells preloaded with labelled DRB, washed and incubated with fresh growth medium showed a release of radioactive material to the medium for up to 24 hr (Fig. 2). Addition of potassium cyanide did not influence the uptake or release of radioactive products significantly (not shown).

Covalent binding of DRB to 10,000 *g* supernatant proteins, mainly sarcoplasmic reticulum and soluble

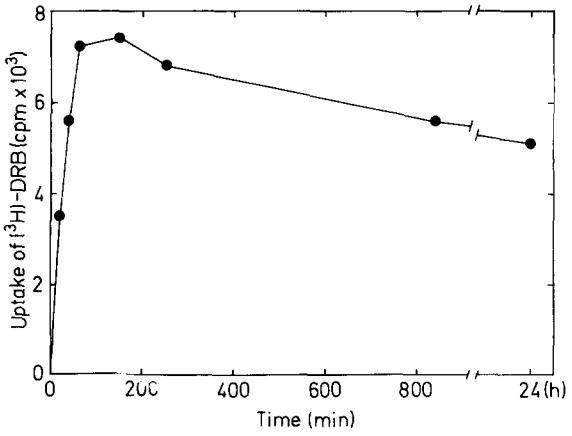

Fig. 1. Accumulation of DRB in heart cells. The cells were isolated and cultured for 3 days as described in Methods, after which 0.85 μM DRB containing 300,000 dpm of (^{3}H)-DRB was added to the medium. At the times indicated the cells were scraped off the dish, washed, collected in 1 ml buffer and counted with respect to radioactivity as described in Methods.

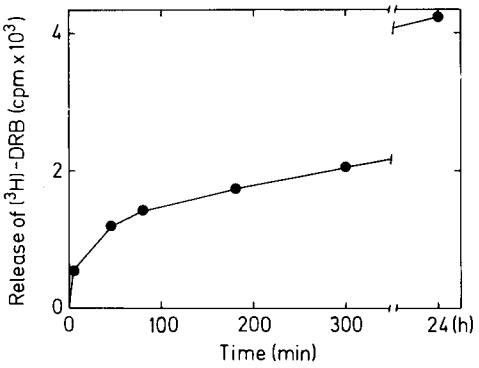

Fig. 2. Release of DRB from preloaded heart cells to the medium. The cells were treated with 300,000 cpm of (^{3}H)-DRB for 24 hr, after which release of radioactivity in 100 μl of medium was measured as described in Methods.

proteins, increased rapidly after addition of 0.85 μM DRB, and reached saturation after about 2.5 hr (Fig. 3A). Estimation of covalent binding to nuclear DNA (Fig. 3B) shows that, in contrast to DRB uptake and protein binding, this binding did not reach saturation but increased linearly for more than 30 hr.

Heart cells treated with DRB at various concentrations and for various lengths of time were investigated with respect to cellular damage by measuring beating frequency and beating amplitude, release of LDH, GSSG and pyruvate to the medium. In short term tests, i.e. for about 2 hr, intermediate concentrations of DRB, e.g. 34 μM, affected the contraction pattern in a manner similar to that observed earlier upon anaerobiosis, i.e. a decreased beating frequency without a change in amplitude (Fig. 4A, cf. also ref. [24]). High concentrations of DRB, i.e. 170 μM or higher, inhibited beating immediately and completely (Fig. 4A). In long-term tests 0.017 μM DRB caused a slight decrease in beating frequency after 7 days (Fig. 4B) without any change in amplitude or morphology (not shown). Cultures treated with 0.17 μM DRB ceased to beat after 2 days although the morphology still appeared normal (not shown). Concentrations of DRB of 1.7 μM or higher had a dramatic effect on the cells, causing complete inhibition within 2 days (Fig. 4B). Also, vacuole appearance, shape changes and partial detachment of the cells from the Petri dish bottom had occurred (not shown).

Release of LDH to the medium appeared to be a less sensitive assay of cell damage. Cells treated with 0.017 μM and 0.17 μM DRB for 2 days released little or no LDH (Fig. 5), although the cells in the latter case had ceased to beat after 1 day. Treatment with 1.17 μM and 1.70 μM DRB led to a pronounced LDH release already from the first day, which reached a maximum after 2–3 days, after which the release was diminished (Fig. 5). Presumably, this transient release of LDH reflected an exhaustion of intracellular LDH after day 2–3 due to cell death. GSSG was detected in the medium after exposure to 1.7 μM DRB (Fig. 6), conditions which simultaneously led to release of LDH. In contrast, release of pyruvate occurred already at a concentration of DRB of 0.17 μM, and thus constitutes a more sensitive assay of cell damage as compared to LDH and GSSG release. Higher concentrations of DRB tended to decrease the release of both pyruvate and GSSG, presumably due to cell death, occurring prior to day 3. Whether the release of pyruvate and GSSG also reflects a leaky plasma membrane, which probably accounts for the LDH release, is presently not known.

The possibility that DRB is metabolically activated by one-electron reduction, catalysed by, for example, NADPH-cytochrome *c* reductase or NADH-ubiquinone reductase, followed by the formation of superoxide, implies that the addition of DRB to the intact heart cell would cause an increased oxygen consumption. Indeed, as may be seen in Fig. 7, 34 μM DRB gave a substantial increase in oxygen

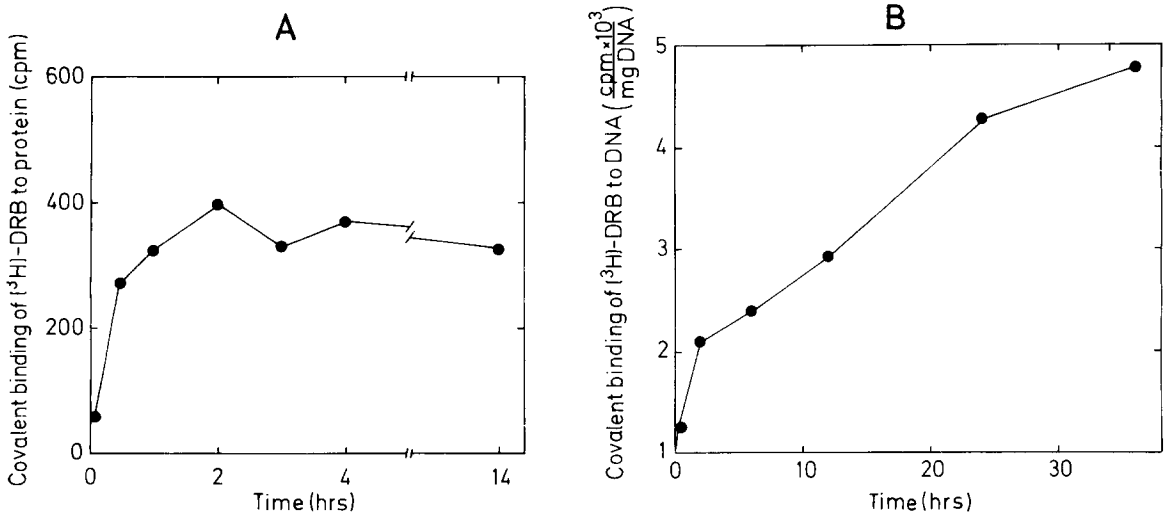

Fig. 3. Covalent binding of DRB to protein (A) and DNA (B) in heart cells. Covalent binding was estimated as described in Methods.

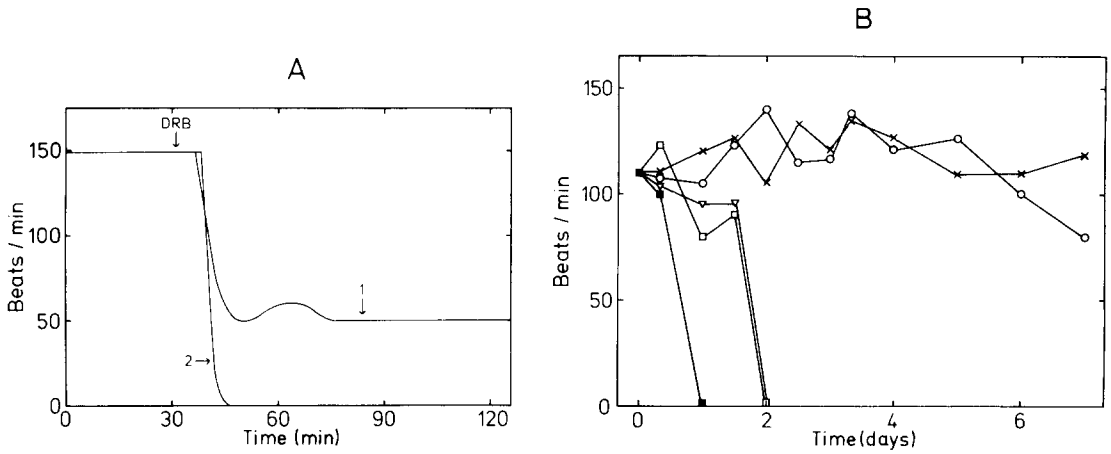

Fig. 4. Short-term (A) and long-term (B) effects of DRB on the beating frequency of heart cells. Beating frequencies were estimated as described in Methods. The concentrations of DRB added were: (A) trace 1, 34 μM; trace 2, 170 μM. (B) ×—×, none; ○—○, 0.017 μM; ▽—▽, 0.17 μM; □—□, 1.70 μM; ■—■, 17.0 μM.

consumption both in the absence (Fig. 7A) and in the presence of potassium cyanide (Fig. 7B). In both cases the addition of DRB caused a peak of oxygen consumption followed by a more constant consumption for at least 1 hr.

DT-diaphorase and glutathione transferase have previously been suggested to contribute to the protection of heart cells from damage induced by DRB by reduction of DRB to the hydroquinone, and conjugation of peroxide products generated, respectively [21]. As shown in Fig. 8, DT-diaphorase is rapidly induced following the addition of DRB and reached maximal activity after 24 hr of treatment. Induction of glutathione transferase was somewhat slower, giving maximal induction after 48 hr of treatment (Fig. 8). This difference in time required for induction is consistent with the conceivable delay in the appearance of peroxide substrates for glutathione transferase. Glutathione peroxidase activity was not influenced by the presence of DRB (not shown).

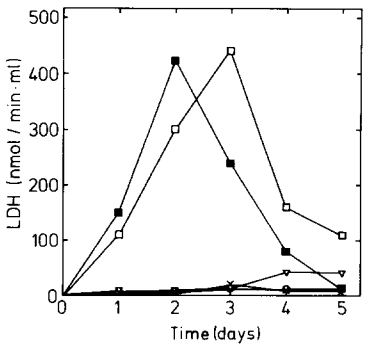

Fig. 5. Release of LDH from heart cells treated with DRB. LDH activity was estimated as described in Methods. The concentrations of DRB added were: ×—×, none; ○—○, 0.017 μM; ▽—▽, 0.17 μM; □—□, 1.70 μM; ■—■, 17.0 μM.

DISCUSSION

The present results show that DRB has pronounced toxic effects on cultured neonatal heart cells from rats. DRB is rapidly taken up by the cells, followed by covalent binding of metabolites to protein and DNA, and, subsequently, cell damage and cell death. The fact that potassium cyanide does not influence uptake or release of DRB indicates that these processes occur by way of passive diffusion rather than active transport. However, at the present time a possible glycolytic generation of ATP cannot be eliminated as a source of energy. In this context it should be stressed that cultured neonatal heart cells survive and continue to beat for at least 3 days in the presence of 1 mM potassium cyanide (not shown). Once taken up by the cells, unmetabolized DRB has been reported to form a complex with cardiolipin [31] and intercalate in DNA [32], which may give rise to both short-term and long-term effects. The bulk of the available information about DRB-induced cardiac damage clearly suggests the involvement of reactive species of DRB. These may be generated either by one-electron reduction to the corresponding semiquinone by, for example, microsomal NADPH-cytochrome *c* reductase [33], mitochondrial NADH-ubiquinone reductase [34], or by various enzymes catalysing reductive cleavage of the sugar moiety, producing alkylating agents [35, 36]. However, most of the conclusions reached so far have been based on results obtained with subcellular systems such as liver microsomes or heart sarcoplasmic reticulum, and it is uncertain whether these conclusions apply *in vivo*.

The selective cardiotoxic effects of DRB make heart cells an ideal model system for studying these effects. However, for various reasons heart cells from adult animals are difficult to keep in culture for more than 24 hr and are therefore unsuitable for this purpose. In contrast, heart cells from neonatal rats can be kept in culture for prolonged periods of time and also respond to concentrations of DRB which

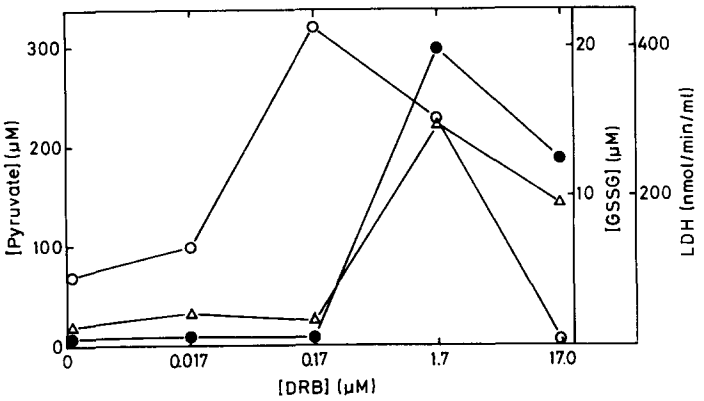

Fig. 6. Release of pyruvate, GSSG and LDH from heart cells treated with DRB. The cells were treated with 0.85 μM DRB for 3 days, after which samples of the medium were taken at the times indicated and analyzed for pyruvate (○—○), GSSG (△—△) and LDH (●—●) as described in Methods.

are of the same order of magnitude as those used in chemotherapy [37]. The latter cells therefore appear to be an attractive alternative system. In terms of sensitivity, changes in beating frequency followed by release of pyruvate, GSSG and LDH, appear to be the most suitable assays for cell damage. However, it is presently difficult to explain why pyruvate is released prior to GSSG. Even though pyruvate in contrast to GSSG is charged, the former is considerably smaller and may be more prone to diffuse through a leaky plasma membrane. In the case of pyruvate a contributing factor may be accumulation due to inhibition of mitochondrial respiration [14], whereas release of GSSG presumably is influenced by the redox state of glutathione and mediated by a specific carrier [38].

No conclusive evidence has as yet been presented which unambiguously demonstrate that DRB toxicity in heart cells or tissue is due to the previously proposed one-electron reduction to a semiquinone followed by reduction of molecular oxygen to superoxide [12]. The demonstration of an increased uptake of oxygen, both in the absence and in the presence of potassium cyanide to block mitochondrial respiration, induced by the addition of DRB, therefore constitutes strong evidence that a free radical pathway is operative in the intact cell which may give rise to lipid peroxidation. It should be emphasized, however, that although DRB-induced oxygen consumption is not conclusive evidence for the initiation of lipid peroxidation by superoxide, it does suggest that semiquinone radicals are formed with the subsequent generation of superoxide and/or lipid peroxides. Whether the semiquinone or the superoxide formed is responsible for a hypothetical lipid peroxidation remains to be shown (cf. ref. [39]). A low

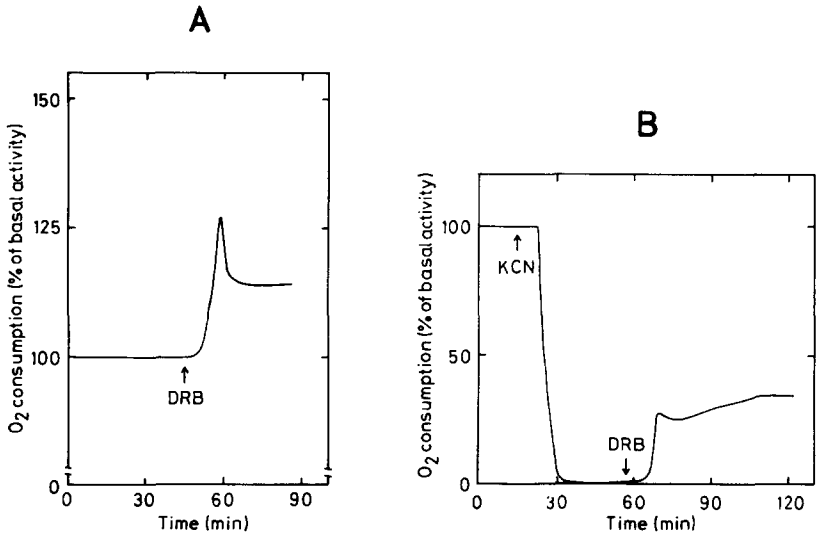

Fig. 7. DRB-dependent increase in oxygen consumption of heart cells in the absence (A) and in the presence (B) of potassium cyanide. Oxygen consumption was measured as described in Methods. Addition were: 34 μM DRB and 1 mM potassium cyanide (KCN).

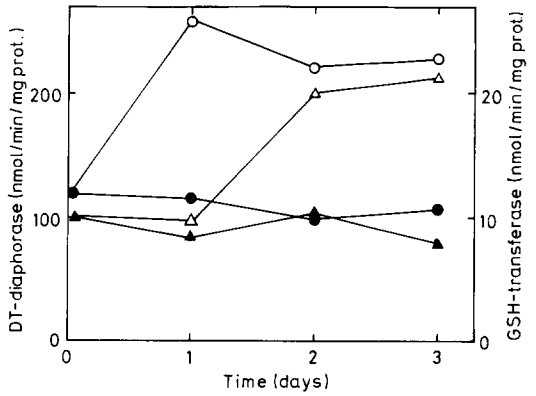

Fig. 8. Time course of induction of DT-diaphorase and glutathione transferase in heart cells treated with DRB. Heart cells were treated with 0.85 μM DRB and the activities of DT-diaphorase (○—○) and glutathione transferase (△—△) were determined as described in Methods. Filled symbols denote control activities.

but significant adriamycin-induced lipid peroxidation has indeed been demonstrated *in vivo* [40]. Thus, it appears conceivable that the existence of a semiquinone pathway in combination with the known relatively low capacity of heart tissue to inactivate hydrogen peroxide and superoxide through catalase and superoxide dismutase, respectively [41], contribute to the high susceptibility of this tissue to damage by radical-generating agents.

The protective role of DT-diaphorase demonstrated earlier [21, 22] indirectly supports the involvement of semiquinone radicals. A rapid induction of DT-diaphorase, demonstrated here, is also consistent with a protective role of this enzyme in DRB toxicity. An interesting observation is that Sudan 3, a potent inducer of DT-diaphorase [42], was without effect on the toxicity of DRB (not shown). This could, however, be explained by assuming that DRB itself is an equally good or better inducer of DT-diaphorase than Sudan 3 (cf. ref. [21]). That glutathione transferase, but not glutathione peroxidase, is induced although to a lower extent than DT-diaphorase, is consistent with the generation of peroxidation products which are conjugated with glutathione [43] and therefore may have an inductive effect on the transferase. These results suggest that the activities of both DT-diaphorase and glutathione transferase, as well as the redox levels of glutathione and NADP, also are important parameters for the heart toxicity of daunorubicin. An interesting possibility is that daunorubicin is activated primarily in the mitochondria [34], in which case mitochondrial radical and quinone-metabolizing systems including the mitochondrial glutathion pool [44] and the NADPH-generating nicotinamide nucleotide transhydrogenase [45] may be important in preventing damage. This possibility is presently being investigated.

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