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DIFFERENT CYTOTOXICITY AND METABOLISM OF DOXORUBICIN, DAUNORUBICIN, EPIRUBICIN, ESORUBICIN AND IDARUBICIN IN CULTURED HUMAN AND RAT HEPATOCYTES*

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Abstract—Both cytotoxicity and metabolism of five anthracyclines, namely doxorubicin, daunorubicin, epirubicin, esorubicin and idarubicin, were investigated in primary cultures of both rat and human adult hepatocytes and, for comparison, in a rat liver epithelial cell line. Toxicity was assessed by morphological examination and measurement of lactate dehydrogenase leakage after 24 hr of treatment. The rank order of toxicity for both rat and human hepatocytes was esorubicin > doxorubicin = epirubicin ≥ idarubicin > daunorubicin, and for rat epithelial cells: esorubicin ≥ epirubicin > idarubicin = daunorubicin = doxorubicin. Human cells were around 2-fold less sensitive than rat hepatocytes to all anthracyclines.

Anthracyclines and their metabolites were analyzed by HPLC. Differences in both the percentages and routes of metabolism were demonstrated between rat and human hepatocytes. The main metabolite was the 13-dihydro-derivative (-ol derivative) in both species from daunorubicin, idarubicin and esorubicin. Glucuronides of epirubicin and epirubicinol were found only in human hepatocytes. In addition, several unidentified metabolites were detected of esorubicin, idarubicin and daunorubicin in rat hepatocytes. In human hepatocytes, only one unknown metabolite from daunorubicin and doxorubicin was found to be formed by cells from a different donor. In spite of variations between individuals, human hepatocytes generally metabolized anthracyclines more actively than did rat hepatocytes. Rat liver epithelial cells were only able to convert daunorubicin and idarubicin, the two molecules which have the best affinity for the non-specific NADPH-dependent aldoketoreductase system. Three compounds (doxorubicin, epirubicin and esorubicin) were present in large amounts in the cells as the parent drug, another (idarubicin) as the 13-dihydro-derivative.

This comparative study on cytotoxicity and metabolism of five anthracyclines in rat and human hepatocyte cultures emphasises species differences and the importance of this *in vitro* model system for further analysis of the metabolism and effect of anthracyclines.

Anthracycline antibiotics are used in the treatment of a wide range of tumors [1–4]. However, their therapeutic usefulness is limited by a number of side effects, one of the most severe being cardiotoxicity [5–9]. Anthracyclines were considered to be cytotoxic by intercalating with cellular DNA [10], or by inhibiting DNA topoisomerase II activity [11]. Another proposed mechanism for their cytotoxic effect involves formation of intracellular free radicals generated by activation of the quinone group of the drugs [12, 13]. These reactive radicals could damage DNA or induce lipid peroxidation in membrane structures [14]. Another study [15] has shown that adriamycin is also cytotoxic in the absence of cell uptake by interacting with the plasma membrane. However, these findings may reflect differences in drug doses, duration of treatment, species or nature of the cells used in the various studies.

Only mild liver cell changes have been demonstrated after anthracycline treatment and they could have been secondary to damage of other tissues. However, daunorubicin was mentioned as a common denominator in 13 cases of suspected drug-induced hepatic injury [16]. Administration of a single dose of doxorubicin decreases the content of cytochrome P-450 and glutathione in rat liver [17] and high levels of glutathione have been found to protect isolated hepatocytes from adriamycin toxicity [18].

All these reports clearly indicate that the mechanisms of anthracycline toxicity are poorly understood. In addition, the rates and routes of metabolism of these antibiotics require further investigations particularly in man. To evaluate metabolism and toxicity of anthracyclines, a limited number of model systems are usually used, including a spectrum of murine and human tumor cell lines. As the liver is the major organ involved in drug biotransformation and the target of toxic effects of

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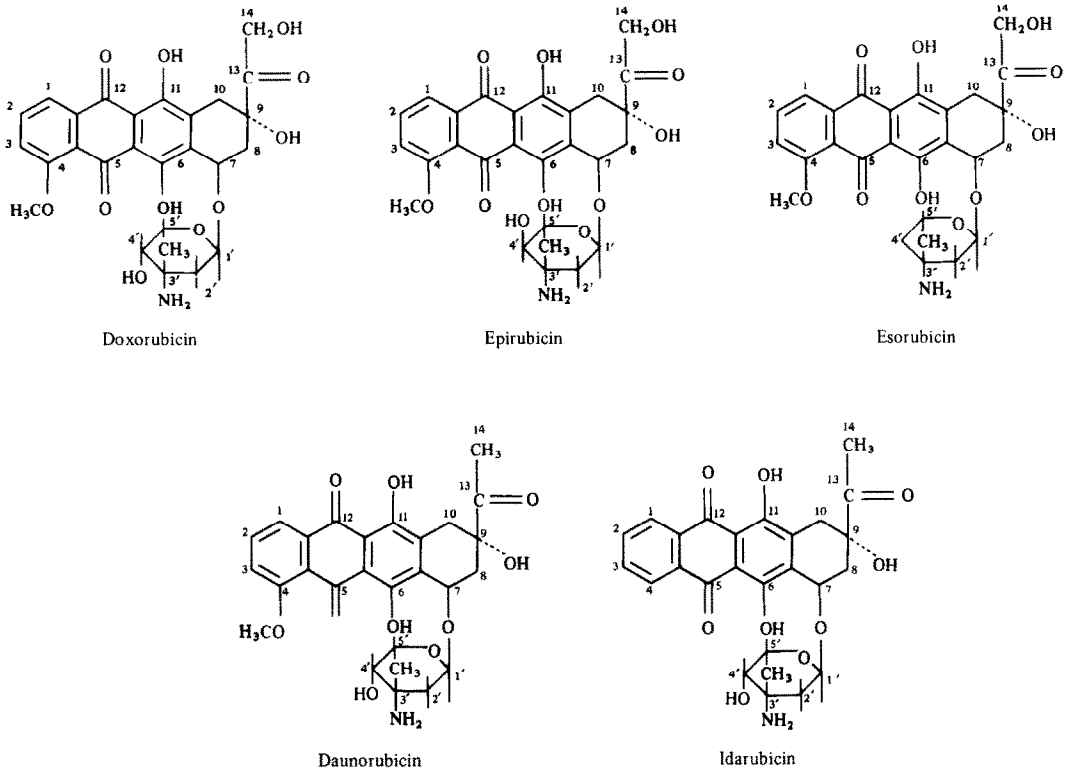

Fig. 1. Structure of the five anthracyclines studied.

many compounds we decided to evaluate cytotoxicity and metabolic pathways of five anthracyclines in primary cultures of both adult human and rat hepatocytes and, for comparison, in an undifferentiated rat liver epithelial cell line. Toxicity studies were, in addition, needed to determine maximum non toxic drug concentrations that could be used for metabolic analyses.

MATERIALS AND METHODS

Chemicals

The following five anthracyclines, namely daunorubicin, doxorubicin, epirubicin, esorubicin and idarubicin were studied. Their structures are given in Fig. 1. They are formed of a chromophore bound to an aminosugar. Daunorubicin was isolated from *Streptomyces peucetius* and doxorubicin from a variant of this strain. Epirubicin is a doxorubicin derivative obtained by replacing the natural aminosugar daunosamine by the corresponding 4′-epi-analog. Esorubicin is a new doxorubicin analog obtained by removing a hydroxyl group at position 4′ of the aminosugar. Idarubicin is a derivative of daunorubicin, which lacks the C-4 methoxy group.

Daunorubicin, doxorubicin and the standard metabolites daunorubicinol and doxorubicinol were kindly supplied by Roger Bellon laboratories (Neuilly, France). Epirubicin, esorubicin, idarubicin and the metabolites epirubicinol, epirubicin glucuronide, epirubicinol glucuronide and idarubicinol were a gift from Farmitalia-Carlo-Erba (Milan, Italy).

Cell isolation and culture

Human hepatocytes were obtained from four male kidney donors aged between 17 and 31 years. The four patients died from traffic accidents, and histological examination revealed normal livers. The protocol for obtaining and using human hepatocytes has been approved by the INSERM ethical committee.

Livers were dissociated using an experimental procedure described elsewhere [19]. Basically, a portion of the left lobe of the liver was rapidly perfused after kidney removal by introducing a canula into a branch of the left portal vein. The liver was first washed with *N*-2-hydroxyethyl piperazine–*N*′-2-ethane sulfonic acid (Hepes) buffer (pH 7.4) then with a Hepes-buffered solution containing 0.05% collagenase.

Cell suspensions were filtered and washed three times by centrifugation. Isolated hepatocytes were seeded either at a density of 5×10^5 cells per 10 cm^2 Petri dish containing 2 ml of medium or at a density of 8×10^6 cells per 75 cm^2 flask in 10 ml of medium. This medium was a mixture of 75% minimum essential medium and 25% medium 199 supplemented with 200 μg/ml bovine serum albumin, 10 μg/ml bovine insulin and 10% fetal calf serum. The cultures were maintained under a 5% CO_2–95% air humidi-

fied atmosphere. The medium, to which 7×10^{-5} M hydrocortisone hemisuccinate had been added, was renewed after 4–8 hr and every day thereafter. Human hepatocyte cultures were used 1–3 days after cell seeding.

Rat hepatocytes were obtained from adult male Sprague–Dawley animals, weighing 180–200 g, by perfusion of the whole liver with 0.025% collagenase as previously described [20]. Culture conditions including medium composition and cell density were those described for human hepatocytes. Rat hepatocyte cultures were used 4 or 18 hr after cell seeding.

Rat liver epithelial cells, probably derived from primitive biliary cells, were obtained by trypsinisation of 10-day-old rat livers and routinely grown in Williams' medium to which 10% fetal calf serum has been added. They were used at confluency around the twentieth passage, i.e. before any sign of spontaneous transformation [21].

Treatment of liver cell cultures with anthracyclines

All the anthracyclines were dissolved in the medium. For cytotoxicity studies various concentrations of each compound ranging between 0.1 and 200 μM were tested over a 24 hr period. The non-toxic concentrations of anthracyclines used in the determination of metabolic patterns were 0.1 μM for esorubicin and idarubicin and 0.15, 0.2 and 0.5 μM for epirubicin, doxorubicin and daunorubicin respectively. To prevent photolytic degradation, all manipulations were performed under subdued light and the cultures were incubated in total darkness.

Evaluation of anthracycline-induced cytotoxicity

Light microscopy. Liver cell cultures were examined under phase-contrast microscopy after 4 and 24 hr of treatment with anthracyclines. Evaluation of the degree of cytotoxicity was based on appearance of dense or refringent intracytoplasmic granules, alterations of the cell shape and disruption of the cell monolayer [22].

Lactate dehydrogenase activity. Quantitative determination of cytotoxicity was assessed by measurement of lactate dehydrogenase (LDH) activity in the culture medium. This parameter has been demonstrated to be a good index of *in vitro* hepatotoxicity [23]. A 100 μl aliquot of the medium was harvested after 4 and 24 hr of incubation with anthracyclines and immediately frozen (−80°) until analysis with a LDH UVP kit system from Boehringer (Mannheim, F.R.G.) using a Cobas Bio-equipment (Roche Analytical Instruments Inc., Nutley, NJ).

To verify the results obtained by the measurement of LDH leakage in the culture medium intracellular LDH activity was also determined.

LDH results were expressed as percentages of content of culture medium or cell extract of untreated hepatocytes.

Identification and quantification of anthracycline metabolites

Parent drugs and metabolites were determined in both culture media and cell extracts. After 24 hr of incubation with anthracycline, the medium was harvested and immediately frozen (−20°). The cell monolayer was washed in phosphate buffer (pH 7.4) and frozen *in situ* until extraction of remaining parent drugs and metabolites.

Extraction procedure. The extraction procedure from culture media was described by Robert [24]. Media were run through C-18 bonded silica (Sep-Pack; Waters Associates, Inc., Milford, MA). After washing with phosphate buffer, the anthracyclines and their metabolites were eluted by 6–12 ml of methanol. The resulting eluate was evaporated to dryness under a stream of nitrogen.

The drugs were extracted from the cells using the procedure of Baurain *et al.* [25] with slight modifications. Cell layers were removed using hypotonic phosphate buffer. Anthracyclines and their metabolites were extracted using a mixture of chloroform/methanol (4/1, v/v) after alkalinization (pH 9.8) with sodium borate 0.5 M. The solid material was removed and the two phases were evaporated to dryness.

High pressure liquid chromatographic (HPLC) analysis

HPLC analysis was performed with a Spectra Physics (San Jose, CA) ternary pump system on 5 μm C-18 bonded silica columns of 125 × 4 mm (Merck, Darmstadt, F.R.G.). Precolumns of 10 μm C-18 bonded silica were purchased from Brownlee Laboratories (Santa Clara, CA). Fluorescence detection was performed using a JASCO FP 210 spectrofluorimeter (Jasco, Tokyo, Japan) with an excitation wavelength of 467 nm and an emission wavelength of 550 nm. Peaks were recorded and measured through an integrator (Spectra Physics, San Jose, CA). The solvent was a mixture of ammonium formate buffer (0.05 M, pH 4) and acetonitrile as described by Israel *et al.* [26]. Either isocratic or gradient elution was used. In the isocratic mode the ratio buffer acetonitrile was 50/50 (v/v) and the flow rate was 1.1 ml/min. The gradient mode was used for better characterization of metabolites. The ratio buffer/acetonitrile was maintained at 80/20 (v/v) for 5 min and then linearly raised to 40/60 in 10 min, and maintained for 5 min, with a flow rate of 1.1 ml/min. Each sample was reconstituted with phosphate buffer (pH 7) and 50 μl was injected into the column through an automatic sampler (Spectra Physics).

Retention times were compared with those of standards of parent drugs and metabolites. Degradation of drugs during the incubation period was evaluated from media incubated without hepatocytes for 24 hr. Determination of fluorescent compound(s) which could interfere with anthracyclines in the analytic procedure was performed by incubating cells in the absence of drugs.

Each metabolite was expressed as the percentage of total fluorescence (100%) which corresponded to unchanged drug and metabolites in both culture medium and cell extract after a 24 hr incubation. In accordance with others [27–32], we assumed that the present drug and metabolites have a relative molar fluorescence of 1. Indeed, only aglycone metabolites, not detected in this study, have been demonstrated to have a higher relative molar fluorescence than the parent drug [31–33].

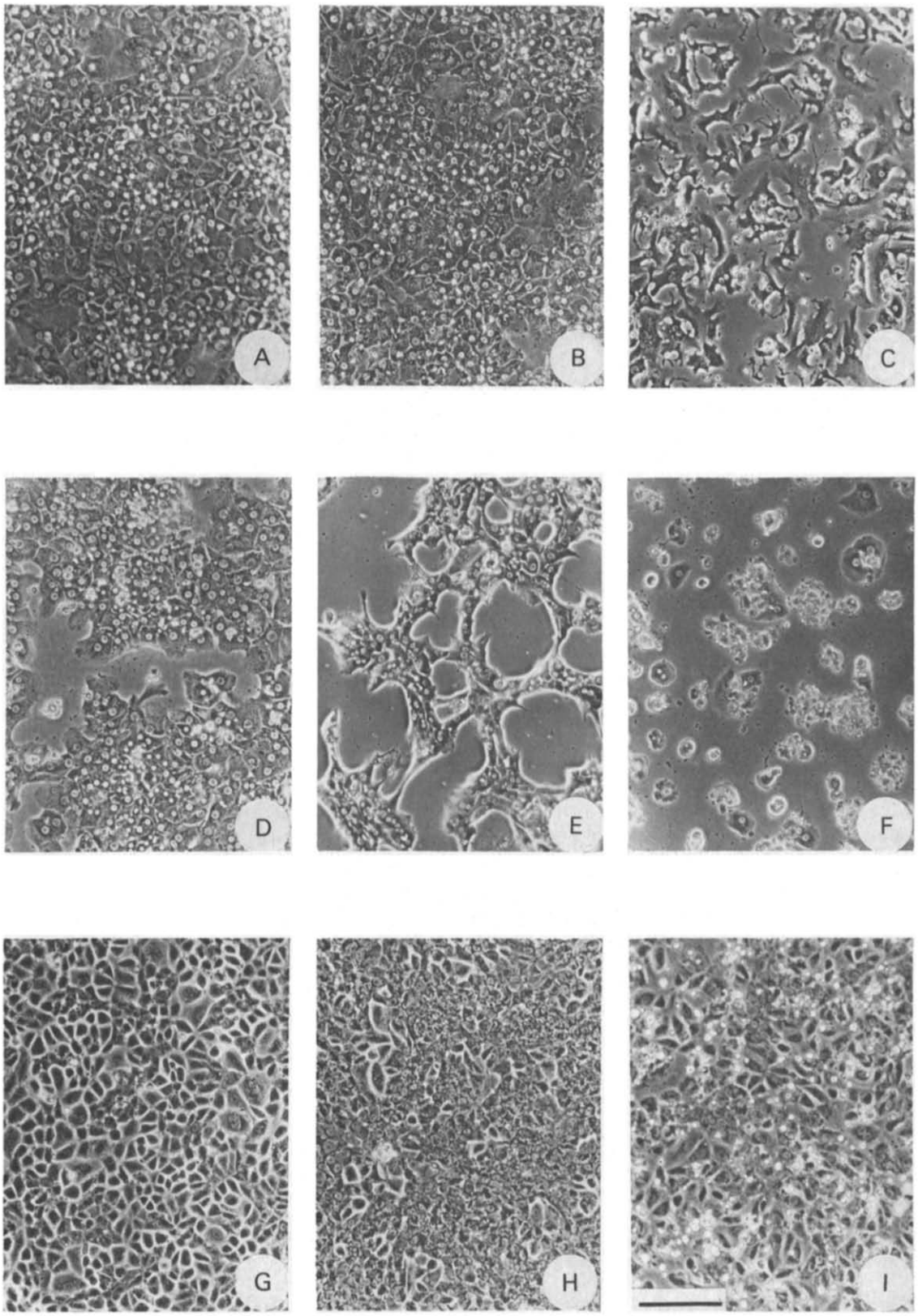

Fig. 2. Morphological examination of rat and human hepatocyte cultures. Human (A,B,C) and rat (D,E,F) hepatocytes, and rat liver epithelial cells (G,H,I) were incubated for 24 hr with 1 μM daunorubicin (A,D,G), idarubicin (B,E,H) and esorubicin (C,F,I), except for daunorubicin which was added to rat liver epithelial cells at 0.1 μM. At 1 μM idarubicin was toxic to rat hepatocytes and liver epithelial cells and esorubicin to the three cell types. Bar, 50 μm. Original magnification, × 130.

RESULTS

Toxicity assessment

Morphological examination. Rat hepatocytes were sensitive to all the compounds. Esorubicin was the most toxic out of the five drugs tested. Marked morphological alterations were induced with 0.5 μM. With other compounds the same degree of toxicity was noticed with 1 or 2 μM. Similar observations were made on human hepatocyte cultures from the four donors. These cells were less sensitive to anthracyclines than their rat counterparts. After a 24 hr incubation, again esorubicin was found to be the most cytotoxic compound. Morphological alterations were visible with a concentration as low as 1 μM. Daunorubicin appeared to be the least toxic drug (Fig. 2). No difference was observed whether rat hepatocytes were taken at 4 or 18 hr and human hepatocytes at 24 or 72 hr.

Confluent rat liver epithelial cell cultures were affected by lower drug concentrations (0.5–1 μM); the most toxic compounds were esorubicin and epirubicin (at 0.5 μM). Marked alterations occurred following treatment with the other compounds at concentrations of 1 μM (not shown).

LDH leakage

Rat hepatocyte cultures. After a 4 hr incubation with various anthracyclines, determination of LDH activity in the medium demonstrated that all the compounds except esorubicin were only slightly toxic at a concentration as high as 200 μM (not shown).

After a 24 hr incubation, esorubicin induced a marked LDH leakage into the medium (× 3.3) even at a concentration as low as 0.5 μM whereas the four other anthracyclines were non toxic at this concentration. The toxic effect of esorubicin was enhanced (× 6.6) at 1 μM. (Fig. 3A). At this concentration doxorubicin and epirubicin induced a 3.4 and 3.8-fold increase in extracellular LDH respectively. Daunorubicin was the less toxic compound: a significant LDH leakage (× 1.4) was evidenced only at 1.5 μM. These results were confirmed by the concomitant decrease of intracellular LDH activity (Fig. 3B).

Human hepatocyte cultures. Whatever the concentration tested, no significant increase of anthracycline-induced LDH leakage into the medium was observed even after 24 hr of incubation. Measurement of remaining intracellular LDH demonstrated that esorubicin was the most potent compound to induce a decrease in intracellular LDH (Fig. 3C).

Rat liver epithelial cells. Measurement of both intracellular LDH content and LDH release into the medium after 24 hr of incubation with various anthracyclines showed that rat liver epithelial cells were highly sensitive to the toxic effect of these drugs. The most toxic components were esorubicin and epirubicin; when incubated at a concentration of 1 μM they induced a decrease in intracellular LDH activity of 39 and 32% respectively. At the same concentration, the LDH decrease was only about 25% with doxorubicin, daunorubicin and idarubicin (Fig. 3D).

Metabolic analysis

Preliminary experiments were performed to evaluate chemical degradation of the five anthracyclines in the culture medium. After a 24 hr incubation in the culture medium about 50% of doxorubicin, epirubicin, esorubicin and idarubicin were degraded while daunorubicin was not modified. In agreement with other studies [33, 34] degradation products had no detectable fluorescent activity; consequently, they were not taken into account for the determination of the total metabolites formed in culture.

Rat hepatocytes. The drugs were added 18 hr after cell seeding. Analysis of the culture medium after 24 hr of incubation demonstrated that rat hepatocytes extensively metabolized idarubicin (85%) and daunorubicin (82%). A lower percentage was found with esorubicin (36%) and only 5% and 6% of respectively epirubicin and doxorubicin were metabolized.

Daunorubicin, doxorubicin, epirubicin and idarubicin were converted into a 13-dihydro-derivative. It is likely that 1 out of 4 unidentified peaks found for esorubicin also corresponded to a 13-dihydro-derivative, namely esorubicinol (Fig. 4). In addition, 3 and 4 unidentified peaks more polar than the 13-dihydro-derivative were observed for daunorubicin and idarubicin respectively. When cell extracts were investigated the percentages of both parent drugs and metabolites were somewhat different from those found in the corresponding media. Differences in the relative amounts of parent drugs and metabolites were also found in cell extracts. Doxorubicin, epirubicin and esorubicin were in greater relative amounts and mostly as the unmetabolized compound (Fig. 5).

Human hepatocytes. Three out of the four cell populations used in toxicity studies were investigated. Human hepatocytes exhibited marked differences from their rat counterparts in both the percentages and routes of anthracycline metabolism. Quantitative, but no striking qualitative, differences were found between the three donors. Human hepatocytes transformed extensively idarubicin (83%), epirubicin (80%), esorubicin (73%), daunorubicin (92%) and to a much lesser extent doxorubicin. Whatever the origin of human cells, idarubicin and esorubicin (Fig. 5) were converted only to their dihydro-derivative, idarubicinol and esorubicinol, while daunorubicin and doxorubicin showed an additional unknown metabolite accounting for 4 and 6% in cases 3 and 1 respectively. In addition, both epirubicin and epirubicinol were glucuroconjugated. Marked variations in the relative amount of anthracycline and metabolites were observed between the three populations of hepatocytes. Doxorubicin and esorubicin were mostly present as the unmetabolized drug; daunorubicin and idarubicin as their reduced 13-dihydro-derivatives (Fig. 5).

Rat liver epithelial cells. These cells were able to metabolize extensively both daunorubicin and idarubicin during a 24 hr incubation period; 82 and 65% respectively were converted into their dihydro-derivative. By contrast, the three other anthracyclines namely doxorubicin, esorubicin and epirubicin remained unchanged. Doxorubicin,

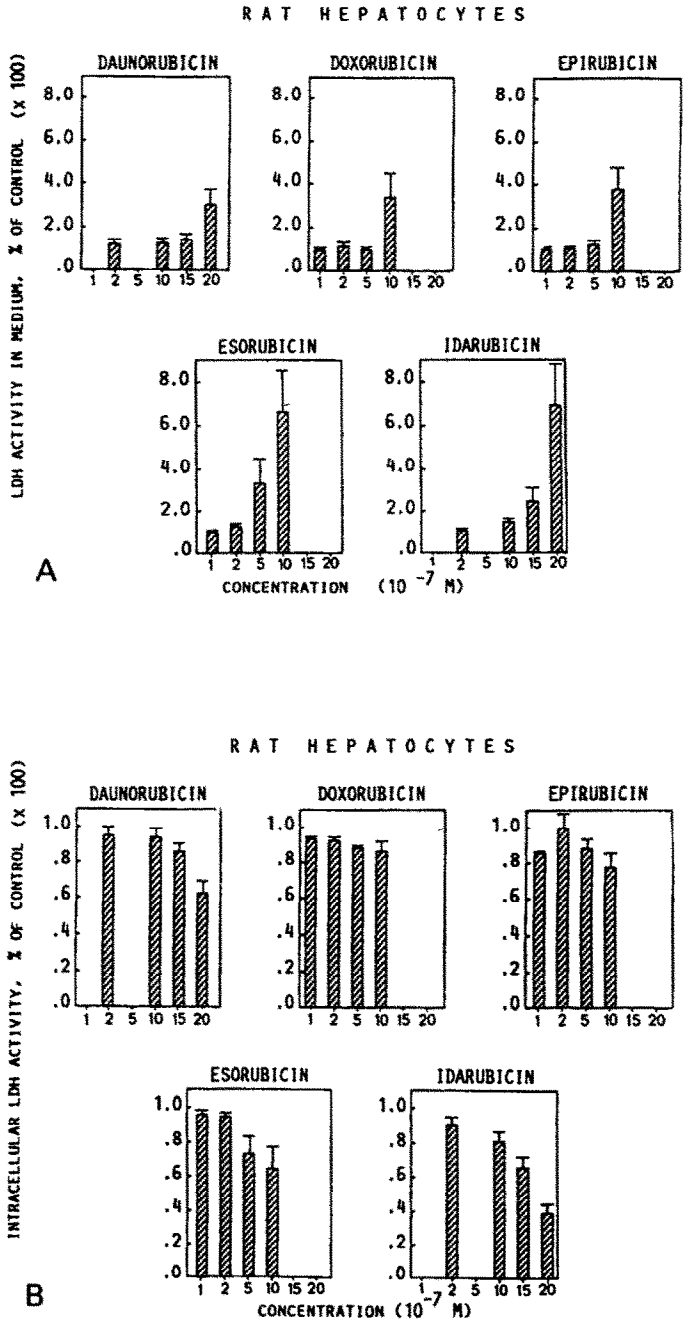

Fig. 3.

epirubicin and esorubicin, which were not metabolized were present in higher amounts in cell extracts than in the medium. For daunorubicin and idarubicin the reduced 13-dihydro-derivative was more abundant than the parent drug (Fig. 5).

DISCUSSION

The present study demonstrates that anthracyclines exhibit some species differences in their degree of cytotoxicity as well as in the percentage of drug metabolized and routes of metabolism in hepatocyte cultures. When compared to rat hepatocytes and rat liver epithelial cells, which do not express specific liver functions [35], human hepatocytes were found to be less sensitive to the five compounds tested. They exhibited no sign of toxicity with all compounds at concentrations lower than 0.5 μM, while both rat hepatocytes and liver epithelial cells were affected by some compounds added at this concentration. However, the same drug, namely esorubicin, had the highest degree of toxicity for the three cell types.

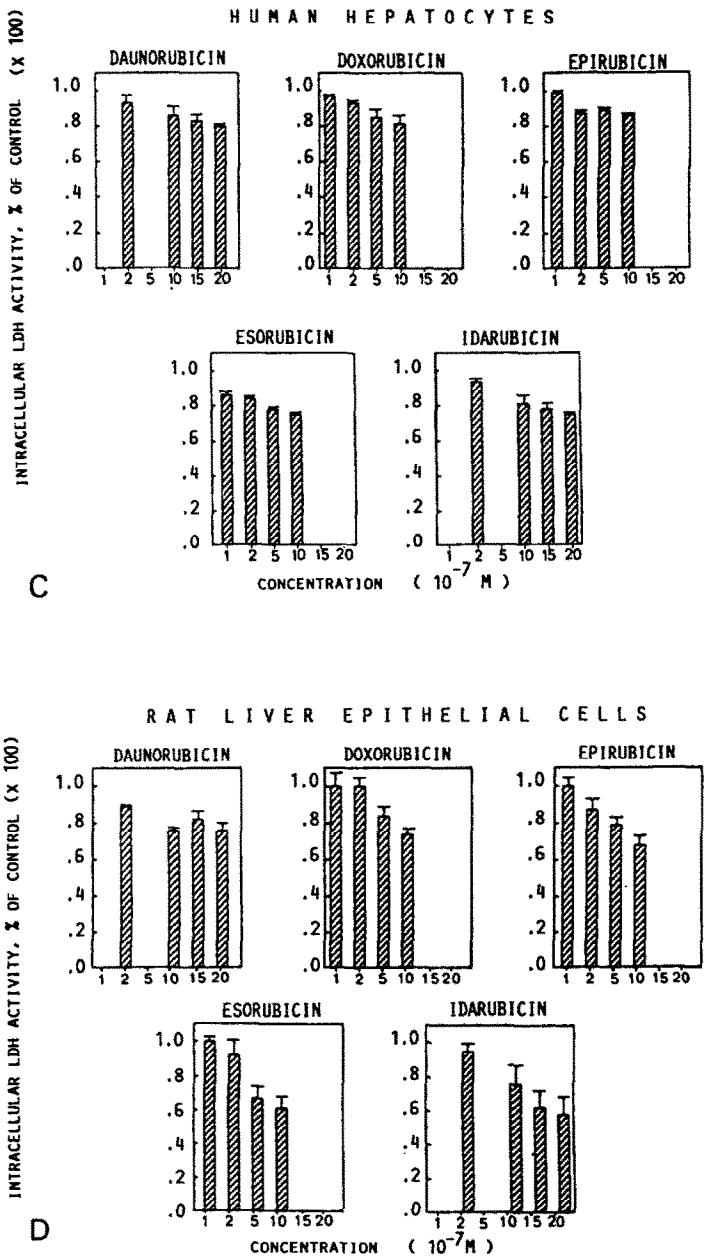

Fig. 3. Comparative cytotoxicity of anthracyclines to rat (A,B) and human (C) hepatocytes and to rat liver epithelial cells (D). Both extracellular (A) and intracellular (B,C,D) LDH activities were measured after 24 hr of incubation with various drug concentrations. Results are means ± SEM of four experiments in duplicate for human and rat cells.

Cytotoxicity evaluation was based on both morphological and biochemical parameters. These gave comparable results. However, light microscopic examination appeared to be more accurate for rat cells while determination of intracellular LDH levels was the most sensitive assay for human cells. Slight morphological changes are more difficult to detect in human hepatocyte cultures since untreated cells already often contain some vacuoles, lipid droplets and/or cytoplasmic dense granules corresponding to secondary lysosomes. The absence of correlation between extracellular and intracellular LDH levels in human hepatocyte cultures is a frequent phenomenon. This observation has been made with a number of toxic compounds and may be due to inactivation or degradation of LDH in the culture medium.

The different sensitivity of the three cell types to the toxic effects of anthracyclines could be either related to differences in functional activity or stability or due to the compound itself, its metabolite(s) [36] or degradation product(s) (34).

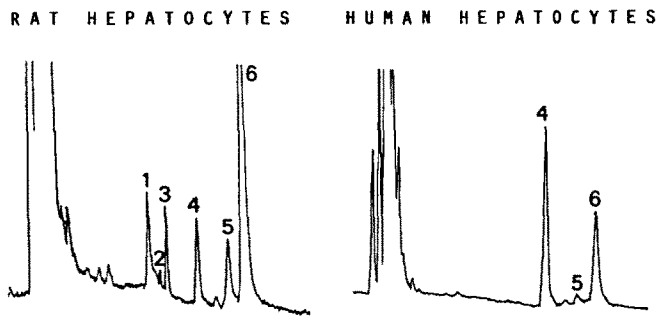

Fig. 4. HPLC chromatogram of medium extracts from rat and human hepatocyte cultures after 24 hr of incubation with 0.1 μM esorubicin. Extraction and HPLC procedure are described in Materials and Methods. Peaks were identified with standards: 1, 2 and 3, unidentified compounds; 4, presumed 13-dihydro-derivative (Eso-ol); 5, unchanged esorubicin; 6, internal standard (daunorubicin).

Previous studies have clearly demonstrated that human hepatocytes survive longer and are functionally more stable than rat hepatocytes in primary culture [37]. As an example, they still express 50–60% of their initial cytochrome P-450 content after 3–4 days [38]. Consequently, a correlation between the degree of cytotoxicity and functional stability is consistent and must be taken into account to explain the different sensitivity of human and rat hepatocytes.

In agreement with *in vivo* observations showing that the liver is the major site of anthracycline metab-

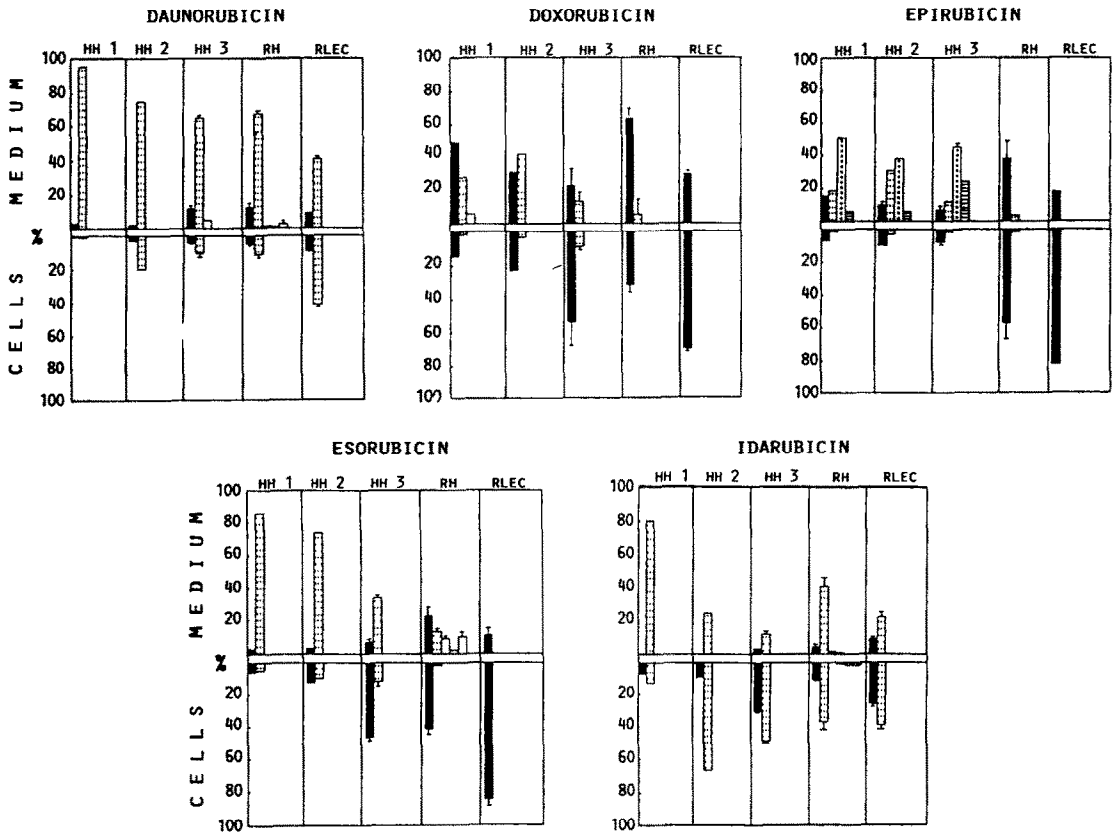

Fig. 5. Comparative metabolism of anthracyclines in rat and human hepatocytes and in rat liver epithelial cells. Intracellular and extracellular percentages of unchanged drugs (■) and metabolites: 13-dihydro-derivatives (▦) and glucuronides of both epirubicin (▨) and its dihydro-derivative (▤) are indicated. (□) represents the percentage of unidentified peaks. Results are means ± SEM of three and four experiments in duplicate for human and rat hepatocytes respectively and two experiments in duplicate for rat liver epithelial cells.

olism, our results demonstrate that both human and rat hepatocytes in primary culture were capable of metabolising the five anthracyclines tested. The major metabolite formed from daunorubicin, esorubicin and idarubicin in both rat and human hepatocytes resulted from reduction on C_{13}. The same observation was made *in vivo* for daunorubicin [39] and idarubicin [40]. By contrast, only traces of epirubicinol were formed by rat hepatocytes while human hepatocytes were not only able to convert this anthracycline into its dihydro-derivative but also to conjugate both of them with glucuronic acid. This finding confirms *in vivo* observations [41] as well as the *in vitro* study on cultured human hepatocytes from one donor reporting appearance of traces of a glucuronide of epirubicin in the medium after a 24 hr incubation with the drug [42]. To our knowledge, the only other studies on isolated hepatocytes reported, as shown here, that rat cells converted daunorubicin and doxorubicin into their dihydro-derivative [31, 32].

Additional qualitative species differences were found for unknown metabolites. Indeed, several unidentified peaks from esorubicin, daunorubicin and idarubicin were demonstrated in rat hepatocyte cultures while only one unknown peak from daunorubicin and doxorubicin was visualized in two different human hepatocyte populations. This finding with human cells could be related either to genetic or environmental factors. Indeed, in addition to genetic polymorphism in drug metabolism that has been well demonstrated in humans, several environmental factors such as premedication and the nutritional status of the donor or conditions of preservation of liver biopsies before cell isolation can influence functional activity of human hepatocytes during the first days of culture [43]. By contrast to previous *in vivo* and *in vitro* studies [31, 32, 44, 45], no aglycone derivatives were detected. However, the formation of these compounds has been claimed to occur only in non-physiological conditions [30, 46]. The discrepancy between freshly isolated cells and cells in culture may be due to the presence of aspecific glycosidases released from lysosomes of cell debris in cell suspensions. It is worthy to note that the intact liver does not produce aglycones [30].

Quantitative variations were found both in metabolism of the different anthracyclines and between the two species. Daunorubicin, idarubicin and esorubicin were more metabolized to the dihydro-derivative than doxorubicin and epirubicin. This is consistent with previous *in vivo* and *in vitro* observations showing that daunorubicin and related compounds which possess a methyl group on C_{14} are better substrates for reductases than doxorubicin and related molecules and does not support the conclusions from one study reporting that esorubicin is poorly metabolized in humans [47]. The same explanation may be proposed for the metabolism of only daunorubicin and idarubicin by rat liver epithelial cells. As a rule, human hepatocytes had a higher metabolic capacity than rat hepatocytes. A previous *in vivo* study with doxorubicin came to the same conclusion [45]. These species quantitative variations can be explained by differences in aldoketoreductase activity. Indeed it has been reported that some human myeloid cell lines contained two families of reductase whilst their rat counterparts have only one [48, 49]. A similar difference could exist between human and rat hepatocytes and would be worth verifying.

The pattern of intracellular and extracellular distribution of anthracyclines and metabolites markedly varied with both the compound and cell type. This could be at least partly explained by the different extent of biotransformation since the greater the metabolic activity the lower the intracellular drug and/or metabolite(s) content. Since anthracycline uptake is passive while their efflux is active, our results on all the anthracyclines except idarubicin could be due to an easier efflux of metabolites compared to unmetabolized drugs.

An interesting finding was that on the contrary of metabolites of other anthracyclines idarubicinol was generally found in much higher amounts into the cells. This suggests that idarubicin is mainly effective after biotransformation. Such an hypothesis has already been proposed [50].

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