selected fragments. Leu⁵-enkephalin induced pulmonary vasoconstriction thus joins a growing list of opioid-mediated responses which are insensitive to naloxone and do not appear to relate to conventional opiate receptor activation.

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Effects of erythromycin derivatives on cultured rat hepatocytes

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Erythromycin and its derivatives are useful antibiotics in the treatment of a variety of human infections. However, these macrolides have some limitations: the base (EB) is inactivated by gastric juice and the estolate (EE) and other derivatives, which are gastroresistant, may produce hepatic injury and jaundice in patients [1, 2]. The mechanisms involved in erythromycin hepatotoxicity are not clear [2]: hypersensitivity of patients and intrinsic toxicity of the drug [3] have been considered.

In rats [4, 5] and humans [6], erythromycins induce biosynthesis of a particular type of cytochrome P-450 which binds their own metabolites, forming an inactive complex with the iron (II) of cytochrome P-450 [7]. Erythromycin derivatives differ in induction of their own transformation and formation of stable complexes [4, 5, 7] but it is not known whether hepatotoxicity and metabolism rate of these compounds are directly correlated.

Experimental *in vitro* models have been used to study the intrinsic toxicity of erythromycin compounds but they have some major shortcomings. Chang liver cells [8, 9] do not express the properties of differentiated parenchymal cells. Freshly isolated hepatocytes [3] surviving only a few hours may respond atypically to chemicals, on account of the morphological and biochemical trauma of isolation. Conventional hepatocyte cultures normally also undergo rapid phenotypic alterations including a large decrease in several drug-metabolizing enzymes [10]. In this regard, hepatocytes co-cultured with rat liver epithelial cells, which maintain various specific liver functions for several days or weeks [11, 12] seem a more reliable experimental model. Pure and mixed hepatocyte cultures have never been used for studying the metabolism and toxicity of erythromycins.

Recently, a new fluorinated derivative of erythromycin ((8S)-8 fluoroerythromycin A) (P-0501A) (EF) [13] was found to be more gastroresistant and more available in animals than the erythromycin base [14, 15]. We used various liver cell cultures to compare the cytotoxicity and cytochrome P-450 complex formation of this new compound with EB and EE.

Methods

Cell cultures: liver cells. Adult hepatocytes were isolated from 2-month-old male Sprague–Dawley rats by perfusing the liver with an Hepes-buffered collagenase solution. Parenchymal cells were seeded in Ham's F 12 medium containing 0.2% bovine serum albumin and 10% foetal calf serum. For the preparation of co-cultures [11], rat liver epithelial cells (see below) were added 3 hr after hepatocyte seeding. Cell confluency was reached within the first 24 hr of co-culture. The medium, supplemented with 7×10^{-5} M or 3.5×10^{-6} M hydrocortisone hemisuccinate for pure and mixed cultures respectively, was renewed every day [11].

Liver epithelial cell lines, derived from 10-day-old Fisher rats by trypsinization of the liver, were used before transformation. These cells did not express specific liver functions [16].

Human liver fibroblasts were obtained by growing liver fragments (from autoptic material of a 3-day-old baby) in minimum essential Eagle's medium containing 10% foetal calf serum [17]. The cells were used after about 10 passages.

Skin fibroblasts. For comparison with human liver fibroblasts, skin fibroblasts (from autoptic material of a 3-year-old infant) were also used after about 10 passages in the same conditions.

Treatments. All erythromycins (from Pierrel S.p.A. Milan, Italy) were dissolved in dimethylsulfoxide (DMSO) and added once to the cultures in concentrations ranging from 1×10^{-4} to 8×10^{-4} M, at various times after cell

seeding: 4, 24, 48 and 72 hr in conventional rat hepatocyte cultures and 4 and 8 days in co-cultures. The DMSO concentration (0.5% of drug solutions) was kept constant and identical to that of controls. After 2.5, 6 and 18 hr of culture with antibiotics, measured amounts (150 μl) of medium were withdrawn, centrifuged at 15,000 g for 5 min to remove cell debris, and assayed for enzyme leakage. All experiments were performed in duplicate. To check for cytochrome P-450 Fe (II)-metabolite complexes, the cells were harvested 24 hr after 3 daily additions of erythromycin compounds at the concentration of 0.5×10^{-4} M.

Assays. Glutamate oxaloacetate transaminase (GOT) and lactate dehydrogenase (LDH) were measured in the culture medium [18, 19]. The activity of these enzymes was not influenced by the drugs examined.

Cytochrome P-450 was determined after homogenizing cells in 3 ml phosphate buffer pH 7.4 containing emulgen [20] according to Omura and Sato [21]. Uncomplexed and total cytochrome P-450 respectively were measured with untreated cell homogenate and after addition of 50 μM potassium ferricyanide for 5 min at 4° [7]. For assays of enzyme leakage in conventional cultures, hepatocytes were seeded in 2 ml medium at the density of 0.5×10^6 cells/9.6 cm^2 Petri dish. To measure cytochrome P-450 concentration, 20×10^6 cells were seeded in 20 ml medium per 175 cm^2 flask. The cell number seeded in co-cultures was half that seeded in conventional cultures.

Protein concentration was assayed as described by Bradford [22] and was not affected by emulgen. The statistical significance of the results was established by two-way analysis of variance and Tukey's test [23].

Results

Cytotoxic effect of erythromycins. LDH leakage into the medium was utilized as an index of cell injury [24]. Similar GOT losses were obtained (not shown). Morphological examination at harvesting showed cytotoxic effect of the drugs: cytoplasmic granulation, blurring of cell borders, shrinking of some cells and disruption of the continuous cell monolayer were observed after 18 hr exposure of 24 hr hepatocyte cultures to 8×10^{-4} M EB. At that concentration, EE killed all the cells while EF and the solvent DMSO alone caused no apparent cell alterations. These observations agreed with LDH leakage.

Toxic effects of erythromycins on pure hepatocyte cultures were correlated with the culture time (Fig. 1): between the 4th hour and the 4th day of culture, LDH leakage was dose-and time-dependent. DMSO had no

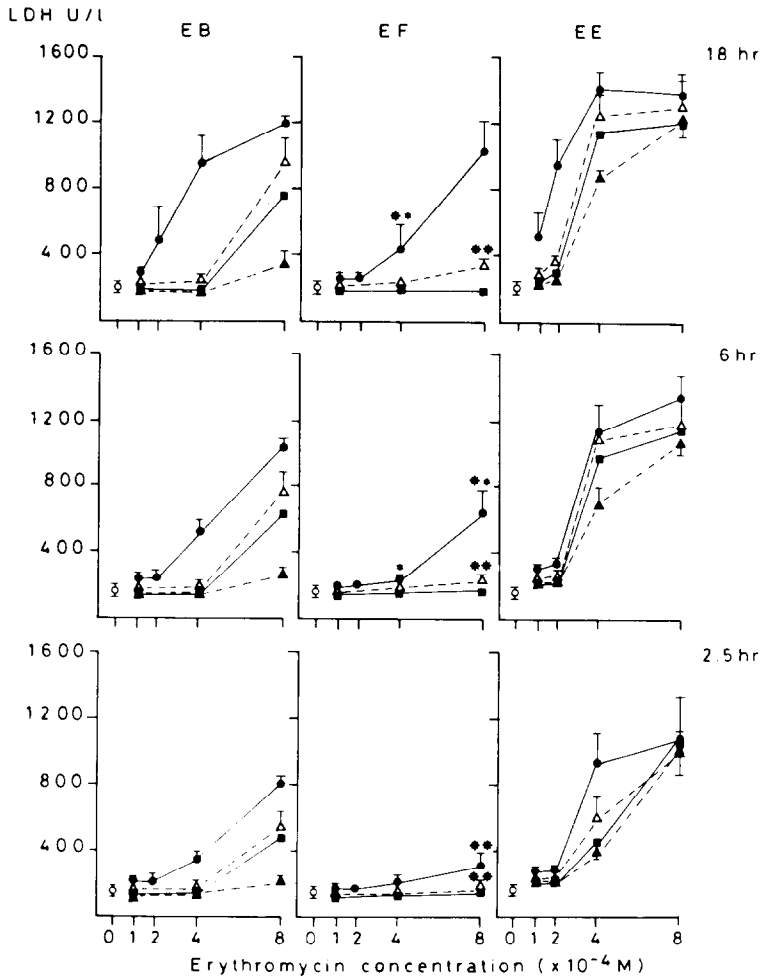

Fig. 1. Time- and dose-dependent effects of EB, EF and EE on LDH leakage from rat hepatocyte cultures. The antibiotics were added at 4 hr (●——●), 24 hr (△---△), 48 hr (■——■) and 72 hr (▲---▲) after plating and LDH was measured at 2.5, 6 and 18 hr after macrolide addition, (o) LDH leakage from control cultures with 0·5% DMSO. Mean values ± S.E.M. of 3 cultures. *$P < 0.05$, **$P < 0.01$ vs the same EB concentration, *$P < 0.01$ vs the same EE concentration.

effect on LDH release and generally toxicity decreased proportionally to culture time, because of lower sensitivity or reduced metabolic capacity of the parenchymal cells. A parallel decline of the cytochrome P-450 concentration was observed during the first 4 days of culture (not shown) [12].

When the antibiotics were added to 4- or 8-day co-cultured hepatocytes which retained a relatively constant level of cytochrome P-450 [12], the same order of toxicity which did not change with the culture time was observed (Fig. 2A).

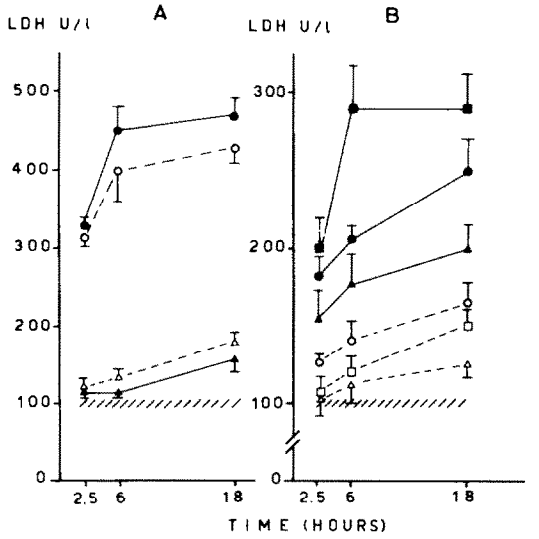

Fig. 2. Time- and dose-dependent effects of EB and EE on LDH leakage from co-cultured hepatocytes (A) and other hepatic and non-hepatic cell cultures (B). (A) 8×10^{-4} M EB (▲—▲, △---△) and 4×10^{-4} M EE (●—●, ○---○) were added to co-cultures at 4 (——) and 8 (– – – –) days after plating and LDH leakage was measured at 2.5, 6 and 18 hr after macrolide addition. (B) 8×10^{-4} M EB (○---○, □---□, △---△) and 4×10^{-4} M EE (●—●, ■—■, ▲—▲) were added to rat liver epithelial cells (○---○, ●—●), human liver fibroblasts (□---□, ■—■) and human skin fibroblasts (△---△, ▲—▲) and LDH leakage was measured at 2.5, 6 and 18 hr after addition. Cells lines were used 3 or 4 days after seeding. LDH leakage from control cultures with 0.5% DMSO (////). Mean values ± S.E.M. of 3 cultures.

Erythromycin cytotoxicity was assessed in other cell types which did not contain detectable amounts of cytochrome P-450, i.e. rat liver epithelial cells, human liver and skin fibroblasts. As shown in Fig. 2(B), EE at the concentration of 4×10^{-4} M was always very toxic while EB was only slightly toxic and EF not toxic at all as in co-cultured hepatocytes (data not shown) at the concentration of 8×10^{-4} M.

Formation of cytochrome P-450 Fe (II)*-metabolite complexes.* In order to clarify the metabolic pathways of erythromycins *in vitro* the induction of cytochrome P-450 by EE and EF and the formation of complexes were studied in co-cultured hepatocytes, which maintain a high cytochrome P-450 concentration in parallel with other specific functions for several days [11, 12].

While 3 daily additions of EF did not affect cytochrome P-450 content in 4-day co-cultured hepatocytes, EE raised the cytochrome P-450 concentration by about 65%. Most of the increased cytochrome P-450 was complexed, presumably with EE metabolites since uncomplexed cytochrome P-450 was not increased by EE treatment (Table 1).

Discussion

The development of stable and non hepatotoxic erythromycin derivatives is of great interest for the treatment of human infections. In order to evaluate cytotoxicity of a new derivative (EF) [13–15] and gain an insight into the general mechanism of hepatotoxicity of macrolides, we have used hepatic and non hepatic cell cultures. Our observations show that EF was not toxic after 18 hr incubation at concentrations up to 8×10^{-4} M while EE was more cytotoxic than EB up to 4×10^{-4} M. These *in vitro* findings with EE and EB agree with *in vivo* results [2].

The cytotoxicity of EB and EE was found to decrease with culture time in conventional hepatocyte cultures. This could be related to decreased metabolism since the concentration of cytochrome P-450 and other specific functions rapidly decline in these cultures [10]. This interpretation is apparently sustained by the absence of decreased toxicity in hepatocyte co-cultures in which the cytochrome P-450 level did not decline [13]. However, in other hepatic and non-hepatic cell cultures, which did not contain detectable amounts of cytochrome P-450, cytotoxicity decreased in the order EE > EB > EF at the same drug concentration as with hepatocytes. Therefore, it may be concluded that *in vitro* cytotoxicity of erythromycin derivatives is not correlated to cytochrome P-450.

The relevance of *in vitro* findings could be questioned since *in vivo* erythromycins are oxidized. Our results with

Table 1. Effect of erythromycin derivatives on cytochrome P-450 content in co-cultured hepatocytes

Cells	Cytochrome P-450 (pmole/10^6 hepatocytes)* Experiment 1	Experiment 2
Control	189	198
Control (+ ferricyanide)	170	175
EE-treated	178	186
EE-treated (+ ferricyanide)	297	276
EF-treated	153	180
EF-treated (+ ferricyanide)	122	152

Cytochrome P-450 concentration was measured with and without 50 μM potassium ferricyanide in homogenates of 4-day co-cultured hepatocytes treated with antibiotics (0.5×10^{-4} M) for 3 days. The values in isolated hepatocytes were respectively 200 and 211 pmoles/10^6 cells in experiments 1 and 2.

* The number of attached hepatocytes was determined before addition of rat liver epithelial cells, in sister pure hepatocyte cultures [12].

hepatocyte co-cultures indicate that EE raised the amount of cytochrome P-450 and induced the formation of stable complexes to the same extent as *in vivo* after 3 days drug treatment of functional hepatocytes, without changes in its toxic effect. In the same conditions, EF did not induce cytochrome P-450 and did not give complexes. This agrees with preliminary *in vivo* findings. These data suggest that hepatotoxicity of erythromycin compounds is not due to prior oxidation of the macrolides.

Our observations are consistent with the statement of an intrinsic toxicity of erythromycin derivatives. However, the primary site of toxicity remains to be determined. This could be the plasma membrane since, in pure but not in mixed hepatocyte cultures, toxicity changed with culture time. Indeed, various alterations of the plasma membrane have been described not only during cell isolation but also in conventional culture [25]. In spite of evident similarities between *in vivo* and *in vitro* effects of erythromycin derivatives, further work is needed to ascertain that the same mechanism is involved in the two situations.

In conclusion, our observations with various liver cell cultures show that: (1) EF is less toxic than EB and EE and does not form stable and inactive complexes with cytochrome P-450; (2) EE is the most toxic erythromycin derivative and induces the formation of an inactive complex with cytochrome P-450. Its toxicity does not seem related to the cells' drug metabolizing capacity; (3) conventional and mainly mixed hepatocyte cultures are useful tools for pharmacotoxicological studies of erythromycin antibiotics and are to some extent predictive of the *in vivo* effects of these drugs.

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Thermodynamics of the interactions of tricyclic drugs with binding sites for [^{3}H]imipramine in mouse cerebral cortex

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The tricyclic antidepressant imipramine, an inhibitor of the neuronal uptake of serotonin, binds with high affinity to specific membrane-associated sites in the central nervous system [1, 2]. A large proportion of these binding sites are located on serotonergic nerve terminals [3, 4], and they recognize drugs with the same specificity as the transporter responsible for serotonin accumulation by nerve endings [5]. Most likely, imipramine and serotonin bind to the same molecular complex involved in serotonin uptake, but at distinct, allosterically interacting sites [6, 7]. Thus, tricyclic drugs would inhibit the uptake of serotonin by inducing conformational changes in the uptake complex [6]. In a