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THE USE OF CULTURED HEPATOCYTES TO ASSESS THE HEPATOTOXICITY OF XENOBIOTICS.

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INTRODUCTION

The liver plays a key role in the metabolism of foreign compounds by modifying their polarity and solubility to facilitate their elimination in a series of reactions known as Phase I and Phase II detoxification reactions. Metabolism of a xenobiotic by the microsomal enzymes in the liver does not mean, however, inactivation in terms of toxicity. Frequently the metabolites originated after transformation by the cit P-450 dependent monooxigenases are more harmful than the original substance and, in many cases, the liver is the first target organ for the reactive metabolites. Hepatotoxicity due to xenobiotics and, in particular to pharmaceuticals, was almost non-existent at the beginning of this century, but today it is becoming a more frequent clinical observation. Animals are used as experimental models in tests aimed at reducing hazards to man for the obvious reason that human beings can not be used in such experiments. However, while there is an increasing public demand for stricter controls of new pharmaceuticals, there is at the same time an increasing social opposition to massive use of laboratory animals to evaluate the risks.

Cellular models have been introduced as complementary and, in part, alternative methods for testing organ specific toxicity. Their use offers the possibility of detecting early cellular changes hardly noticeable in animal experiments (1-3). Freshly isolated and cultured hepatocytes had been previously used to study the hepatotoxicity of drugs (4-9), but isolated hepatocytes can be only used in 2-3 h experiments (8,10). Hepatocytes in monolayer culture retain most of the specific capabilities of liver (11-14), reproduce to a large extent the biochemistry of the intact liver, and may therefore represent a simplified model for studying the hepatic effects of xenobiotics. In addition, cultured cells allow experiments with longer exposition to the xenobiotics. The use of primary cultures of hepatocytes seems to be a promising alternative for the early detection of hepatotoxic effects of xenobiotics (7-9).

Several attempts have been made to establish experimental protocols for the screening of hepatotoxicity of compounds (3-5,15,16). In previous research only cytotoxic parameters (cell viability, cell survival, enzyme leakage etc.) were assessed. This certainly represents one possible approach to a general screening

protocol but may leave out of consideration substances that impair cell function without causing cell death. Such an effect, although not critical for the hepatocyte itself, is of toxicological significance, since the general homeostasis of the organism can be indirectly altered as a consequece of the hepatocyte malfunction. The detection of this early cell injury produced by xenobiotics and its reversibility is desirable but at the same time extremely difficult to assess "in vivo". An "in vitro" model aimed at evaluating this early hepatotoxicity of xenobiotics has to take into consideration this possibility.

In our laboratory, a screening test consisting of an integrated series of experiments to evaluate the action of a xenobiotic on the biology and biochemistry of the hepatocyte has been developed and used in several studies (17-19). In developing the "in vitro" assay for hepatotoxicity, several facts were taken into consideration: first, the use of a biological system that should reproduce to a great extent the biochemistry of the liver; second, the choice of the most appropiate citological/biochemical parameters for detecting and quantifying the hepatotoxic (cytotoxic or cholestatic) effects of the xenobiotic in culture; and finally, the predictive value of the data obtained "in vitro" in relation to the expected and/or observed toxicity "in vivo". First, we examine the cytotoxic effects by measuring cell viability, attachment index and spreading, ultrastructure and morphology, cell monolayer formation and survival, and loss of cytosolic enzymes. Then, the effects on hepatocyte metabolism, namely, gluconeogenesis, glycogen metabolism, ureogenesis, plasma protein synthesis and enzyme induction, are evaluated. The results of the experiments provide information about the kind and degree of interference in liver metabolism caused by a particular xenobiotic. In this paper we present the cytotoxic and metabolic effects observed after exposing cultured hepatocytes to well established indirect hepatotoxins.

MATERIAL AND METHODS

Isolation and primary culture of hepatocytes.

The hepatocytes were obtained from 200-250 g S&D male rats fed ad libitum. The liver was perfused with collagenase as described in detail elsewhere (20). Hepatocytes with viabilities greater than 85% were seeded onto plastic culture dishes previously coated with fibronectin (21). Cells were serum-free cultured in Ham's F-12, supplemented with 0.2% bovine serum albumin, 10^{-8}M insulin and penicillin /streptomycin . The medium was renewed 1 h after plating to remove non attached cells. The medium was then renewed daily and 5 x 10^{-8}M dexamethasone was added to the culture medium.

Cytotoxicity indices.

Cell viability was estimated by the dye exclusion test with 0.027% Trypan Blue in saline and was measured 1 and 24 hours after the addition of the xenobiotic. Cell attachment was measured in plates where the xenobiotics were added at the time of plating. The efficiency of cell attachment was, on an average, 74±6% of an inoculum of 1.75 x 10^6 cells/plate. This corresponds to a final cell density of 65.000 cells/cm^2. To this value we assign an attachment index (AI) of 100. The attachment index in cultures exposed to xenobiotics was measured as we described elsewhere (21) and calculated in reference to this maximal cell density. Hepatocyte spreading on plates was evaluated during the first 6 hours after cell plating in presence of the xenobiotic on micrographs obtained at set-time periods in an inverted phase contrast microscope. Leakage of the intracellular enzymes glutamate oxalacetate trasaminase, GOT (22), and lactate dehydrogenase, LDH (23),was measured in the medium after 6 and 24 hours of treatment with the xenobiotic. Protein was determined by the method of Lowry using BSA as standard. The ultrastructure was studied after 24, 48 and 72 hours of continous exposure to the xenobiotics. Hepatocyte monolayers were fixed in situ with 2% glutaraldehyde and 5% sucrose in 0.1 M sodium cacodilate buffer pH 7.2, and post-fixed with 1% OsO_4 in 1.8% potassium ferrocyanide in phosphate buffer. Finally, cells were included in Epon and sections were stained with 1% uranyl acetate.

Metabolic determinations.

The metabolic parameters were evaluated in 24 hour cultures after a variable pre-treatment with the xenobiotics. Unless specified, the xenobiotics were also present during the assay. The gluconeogenesis and the glycogen content of cells, the rate of urea production during 120 minutes after a load with ammonium, and the albumin synthesis rate during 4 hours were measured as described (17). The activity of aryl hydrocarbon hydroxylase, AHH(23), and 7- ethoxycumarin O-deethylase, ETC (25), were measured in the microsomal fraction of the cytosol (10000g 20 min) after 24 and 48 hours induction either with 1.85 μM 3-methyl-cholanthrene or 2 mM phenobarbital as inducers. To measure the conjugation of 4-nitrophenol and 3-methyl-umbelliferone with UDP-glucuronic acid, hepatocyte monolayers were incubated with 0.15 mM and 0.1 mM of the substrata respectively. Aliquots of the culture medium were taken at set time periods and the remaining concentration (i.e. non-conjugated) of 4-nitrophenol (max 405 nm e=18500 l x mol^{-1} x cm^{-1}) or 3-methyl-umbelliferone (excitation 376 nm, emission 460 nm) was calculated. RNA synthesis was studied by measuring the kinetics of uptake by cells and the incorporation into acid-insoluble material of ^{14}C orotic acid (10 μM, specific activity in the assay 30 μCi/μmol) as described (26).

RESULTS AND DISCUSSION

The "in vitro" hepatotoxicity screening protocol.

As summarized in Fig. 1, the strategy of the screening protocol involves exposing the hepatocytes in cultures to the xenobiotic at different concentrations for variable periods of time. Then two types of effects are evaluated: 1) cytotoxic effects during the initial stages of culture prior to the formation of the confluent monolayer, for the purpose of determining the highest concentration of xenobiotic compatible with hepatocyte survival, and 2) effects on the metabolism and specific liver cell functions of the xenobiotic treated cells at sub-lethal concentrations.

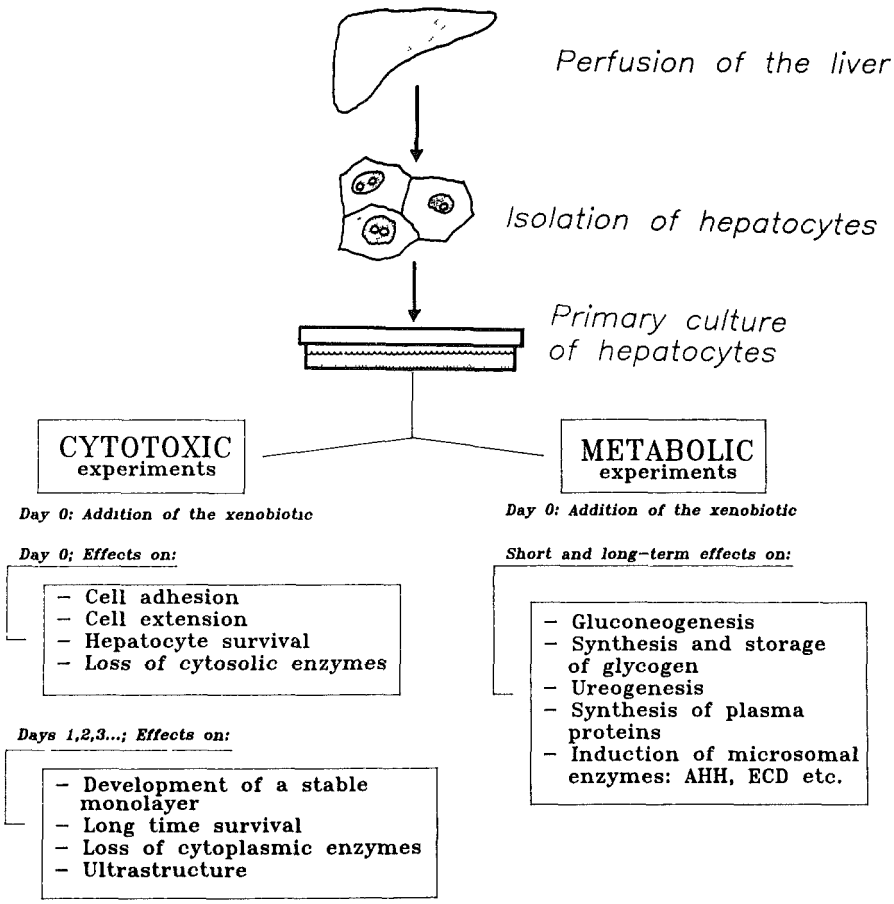

Fig. 1. Schematic diagram of the assaying protocol for hepatotoxicity in cultured hepatocytes.

Cytotoxic effects of hepatotoxins on cultured hepatocytes.

Shortly after cell plating, hepatocytes enter into contact with the fibronectin coated plastic dishes and after an initial lag of about 10 min attach to the plates, showing sigmoid kinetics and reaching the maximum (65,000 cells/cm^2) after 1 hour incubation at 37ºC (Fig. 2A). We then compare the attachment of cells in the presence of the xenobiotic. With this assay the cell-attachment kinetics of xenobiotic-treated cells in relation to controls are evaluated. This fibronectin-mediated attachment of hepatocytes is an active process that is highly selective for viable cells (21). It can be disturbed by direct alterations of the cell membrane (i.e. alteration of the functionality of the receptors for fibronectin), or indirectly, when a compound causes hepatocyte cell death. As shown in Fig 2B there is a good correlation between AI and cell viability. This behaviour observed in cultures treated with cytotoxic concentrations of xenobiotics is accompanied by extensive enzyme leakage.

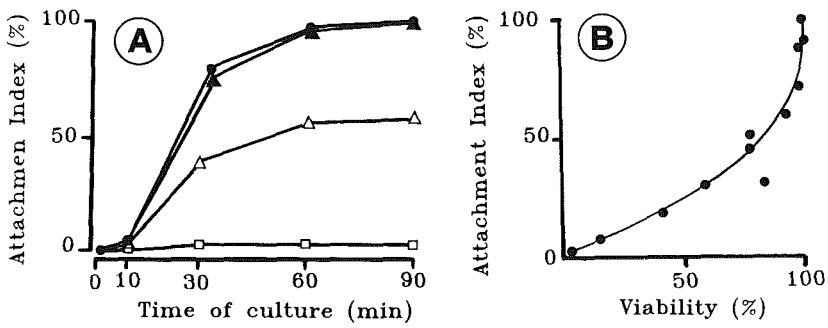

Fig. 2. A) Kinetics of hepatocyte attachment to fibronectin-coated culture plates. (●) Control; Incubated with 1mM (Δ) and (▲) 0.1 mM butibufen, NSAID drug of the phenylalkanoic serie; (□) Dead hepatocytes according to the trypan blue dye exclusion test. B) Correlation between attachment index and viability of hepatocytes treated with several xenobiotics.

An important requirement for developing a stable hepatocyte monolayer in culture is the spreading of cells on the substrata. Hepatocytes, once attached, are very restrictive in their environmental requirements for spreading substratum, because during approximately the first two hours, RNA and protein synthesis later followed by cytoskeleton rearrangement are involved in this process (27). Consequently, cell extension can be strongly influenced by the presence of xenobiotics. After 2 hours in culture, control hepatocytes begin to flatten out until contact is made with neighbouring cells (6-8 h), resulting in confluent monolayers (Fig. 3).

The presence of compounds that inhibit RNA synthesis, protein synthesis or cytoskeleton rearrangement only blocks hepatocyte spreading if these compounds are present during the first two hours of culture. When added later, cells are not so sensitive and spreading may apparently be accomplished by 8 h (27). Blockage of cell spreading during the first stages of culture indicates impairment of one of the processes normally involved in the cell spreading. Cultures are again examined after 24 of seeding. Control cultures then show a characteristic polygonal-shaped frame of cells covering the surface of the plate. Monolayer formation is not accomplished if the xenobiotics have disturbed any of the initial steps of culture. Also, in the cases in which no noticeable effects are observed during the first 6-8 hours after plating, the examination of the monolayer after 24 h is a more sensitive parameter and by affecting, for instance, cellular energetics may indirectly reveal an action of the xenobiotic on other cellular processes.

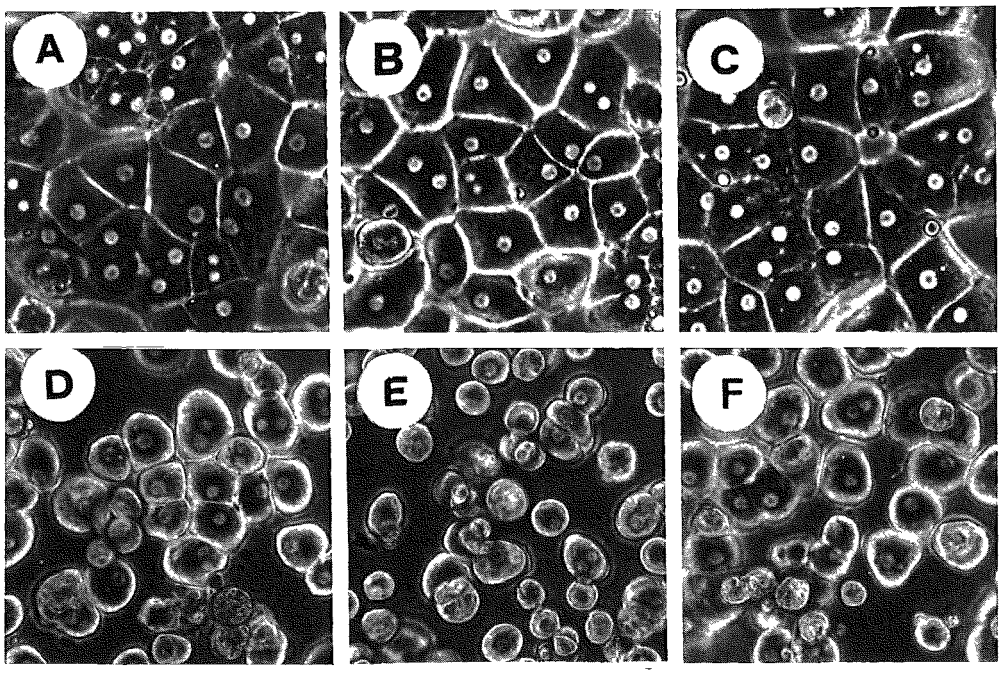

Fig. 3. Effect of metabolic inhibitors on hepatocyte spreading on fibronectin-coated plates. Pictures were taken 6 h after cell plating. Inhibitors were added 2 h after cell plating (A-C), or at the time of cell plating (D-F). Actinomicin D (A,D); Cycloheximide (B,E); Colchicin (C,F).

The ultrastructural study of hepatocytes reveals cellular damage in organelles caused by cytotoxic effects even when cells do not show any apparent morphological injury at the optical level. However, in spite of the systematic work done by other authors in the evaluation of ultrastructural damage (28), the quantification is still difficult to perform and subjectively evaluated, and remained, in our experience, as a useful but qualitative parameter. Ultrastructural changes in organelles were evident in 72 hour cultures that had been exposed to 1 mM acetaminophen for 48 hours. Lower doses did not appreciably alter the ultrastructure of cultured hepatocytes. These concentrations of acetaminophen affected neither hepatocyte viability (1 and 24 hours), nor attachment, nor cell spreading on the plates.

The release of cytosolic enzymes in the culture medium is a more sensitive parameter and has been widely used as an indicator of increased membrane permeability and, indirectly, as a marker of "in vitro" toxicity (5,29). Among the enzymes used in our laboratory, LDH and GOT were more sensitive parameters than GPT. The extent of the enzyme leakage depends both on the concentration and on the time of exposure to the xenobiotic. Frequently, cytotoxicity is indirectly evaluated by the trypan blue dye exclusion test. This technique gives only a rough approximation of membrane damage because only seriously altered (i.e. dead) cells will not exclude the dye and stain. Exposure of hepatocytes to 400 μM galactosamine for 6 h does not affect either viability and only moderately increase LDH leakage. However, after 24 h exposure, much lower concentrations (e.g. 4 μM) increase the enzyme leakage by 27% (Table 1) without affecting cell viability. In our experience the loss of intracellular enzymes normally preceeded other cytotoxic effects (i.e. cell survival expressed as cellular protein still attached to plates).

TABLE I

LDH LEAKAGE AND CELLULAR PROTEIN CONTENT IN HEPATOCYTE CULTURES TREATED WITH D-GALACTOSAMINE

Concentration of Galactosamine	6 hours exposure		24 hours exposure	
	LDH activity (mU/mg x min)	Cellular prot. (mg/plate)	LDH activity (mU/mg x min)	Cellular prot. (mg/plate)
4 mM	51.8 ± 8.2 (140%)	2.7 ± 0.1	764 ± 92 (898%)	1.6 ± 0.1
0.4 mM	37.6 ± 2.7 (102%)	2.8 ± 0.1	210 ± 39 (247%)	1.9 ± 0.2
40 μM	36.5 ± 2.6 (100%)	2.6 ± 0.1	110 ± 4.6 (129%)	2.1 ± 0.1
4 μM	38.6 ± 2.8 (105%)	2.3 ± 0.05	108 ± 2.8 (127%)	2.1 ± 0.07
Control	36.5 ± 1.4 (100)	2.6 ± 0.2	85 ± 7.4 (100)	2.1 ± 0.17

Acetaminophen is a potent depletor of hepatic GSH. After the levels of GSH fall below a certain level, irreversible cellular damage occurs that is accompanied by cell membrane damage. Leakage of LDH was evident at 0.1 mM, as already reported (30,31). The leakage of GOT increased by 42% in cultures treated with 10 mM acetaminophen, while lower concentrations (0.1 mM) already produced a 35% increase in LDH.

Metabolic effects of hepatotoxins on cultured hepatocytes.

After a confluent monolayer of hepatocytes has been formed, cells recover their functionality and behave as mature hepatocytes, showing the typical functions of a liver parenchymal cell (11-14). It is at this point that changes in the metabolic response or impairment of liver functions by sub-lethal concentrations of xenobiotics can be more accurately measured. As previously stated, the aim of the cytotoxic experiments is to define the concentration of a xenobiotic that is incompatible with cell survival and, hence, to study impairment of metabolism at concentrations below this sub-lethal level, at which cytotoxic effects would not normally be detected but interferences with hepatocyte metabolism may occur.

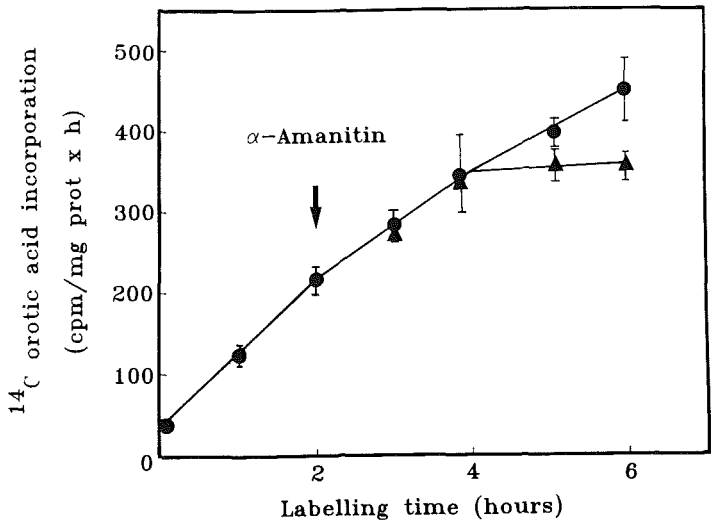

Fig. 4. Time course of ^{14}C orotic acid incorporation into acid insoluble material by 24-hour hepatocyte monolayers. Monolayers were labeled for the indicated times with the tracer at a final specific activity of 30 μCi/umol. a-Amanitin was added two hours after the beginning of the experiment (arrow). (●) Control; (▲) 1.5 pg/mg cell protein a-amanitin. Each point represents the mean of triplicate samples.

a-Amanitin is a strong inhibitor of RNA polymerase-II and a potent hepatotoxin (32,33). a-Amanitin in the range 1.5 ng/ml to 1.5 pg/ml showed no effect on the 1 hour viability and attachment tests. In contrast, at the highest concentration it clearly altered cell spreading and the development of the monolayer. When added to cultures at concentrations of 1.5 pm/ml it inhibited the incorporation of ^{14}C orotic acid in hepatocytes (Fig.4). The incorporation of the tracer into control hepatocytes was linear for 6 h. When a-Amanitin was added 2 hours after the addition of the tracer the inhibition of the RNA synthesis was, as fig. 4 shows, clearly noticeable 2 hours later. The lowest concentration represented in this experiment, 1.5 pg/ml, inhibited the RNA synthesis by 93%. The synthesis of albumin was equally affected. However, a longer exposure time to a-amanitin was necessary. Initially the albumin synthesis rate was unaffected after 7 hours (after 3 h pre-treatment and 4 h assay the albumin production by hepatocytes was 1.69±0.12 μg/mg x hour, 97% of control) but after 13 h pre-treatment the albumin production fell to 0.94±0.09 μg/mg x hour, 56% of that of control. Consequently, 17 hours after exposure, when the mRNA levels had diminished, the synthesis of albumin was inhibited by 39% in relation to controls.

D-galactosamine produces marked changes in the concentration of uracil nucleotides UMP, UDP and UTP and UDP-hexoses "in vivo" (34,35). As a result, an impairment in the synthesis of glycogen, RNA and protein synthesis and glucuronidation is to be expected. This is, in fact, what was observed in cultured hepatocytes exposed to 4 μM galactosamine (Table II and fig. 5).

TABLE II

TOXIC EFFECTS OF D-GALACTOSAMINE ON CULTURED HEPATOCYTES

Concentration	Glycogen content[a] (nmol/mg)	Glucuronidation (pmol/mg x min)	Albumin synthesis[b] (μg/mg x h)
4 mM	52.2 ± 5.9 (50%)	353 ± 15 (50%)	1.9 ± 0.2 (72%)
0.4 mM	75.4 ± 9.1 (73%)	542 ± 18 (76%)	2.5 ± 0.2 (91%)
40 μM	71.3 ± 8.7 (69%)	618 ± 23 (87%)	2.6 ± 0.2 (95%)
4 μM	73.0 ± 8.8 (70%)	710 ± 31 (100%)	2.6 ± 0.2 (95%)
Control	103.8 ± 8.8 (100)	713 ± 28 (100)	2.7 ± 0.2 (100)

(a) Glycogen was measured in 24 h cultures, exposed for 14 h to the drug after stimulation with 10^{-8} M insulin. (b) Albumin was assayed by ELISA in culture medium. Aliquots were taken along 4 h exposure period to the drug, and from this, the rate of albumin excretion was determined.

Galactosamine at the highest concentration assayed (4 mM) did not significantly affect either viability (1 and 24 h exposure) or attachment, nor cell spreading or monolayer development. The synthesis of glycogen by hepatocytes cultured in 10 mM glucose upon stimulation with 10^{-8}M insulin was greatly reduced after 4 h exposure of cells to 4 μM galactosamine (Table II). Also, the conjugation of p-nitrophenol and 3-methyl umbelliferone, which is dependent on the availability of UDP-glucuronic acid, was impaired (0.542 nmol p-nitrophenol/mg x min versus 0.713 nmol/mg x min) at 0.4 mM galactosamine (Table II). For 3-methyl umbelliferone the effects were observed at 4 mM galactosamine (0.252 nmol/mg x min versus 0.536 nmol/mg x min). The inhibition of the albumin synthesis (28%) was observed at 4 mM after 4 h exposure. The incorporation of ^{14}C orotic acid into cells was reduced after 4 h treatment with 0.4 mM, as shown in Figure 5, but a concentration of 4 mM greatly reduced the uptake and the incorporation of the tracer from the beginning of the experiment. Thioacetamide produces "in vivo" a reduced flux of urea formation with marked accumulation of ammonia, both of which

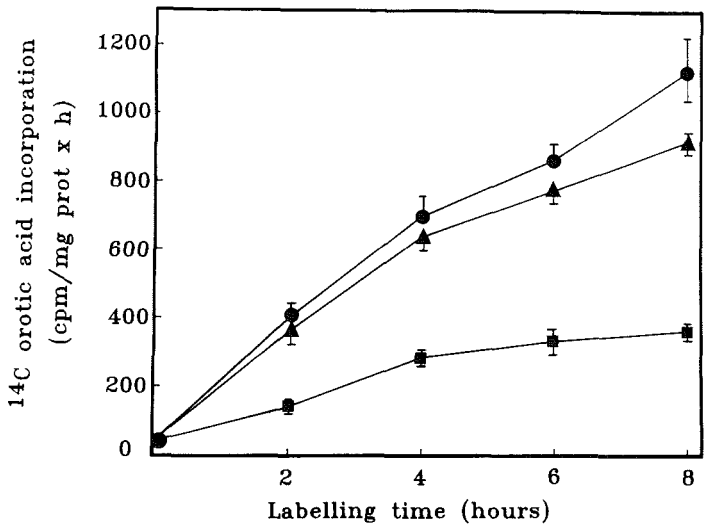

Fig. 5. Time course of ^{14}C orotic acid incorporation into acid insoluble material by 24-hour hepatocyte monolayers. Monolayers were labeled for the indicated times with the tracer at a final specific activity of 30 uCi/umol. D-Galactosamine was added at the beginning of the experiment. (●) Control; (▲) 0.4 mM; (■) 4 mM D-galactosamine. Each point represents the mean of triplicate samples.

are recognized symptoms of liver failure (36,37). This was also observed in cultures after exposing hepatocytes to 2.3 μM thioacetamide for 48 h. However, no cytotoxic effects during the early steps of culture were observed after exposure to 230 μM thioacetamide. The monolayer developed as did the controls and survival was not impaired after 4 days. A 31% inhibition of the urea production by hepatocytes was observed by 24 hours of exposure to 230 μM thioacetamide (control urea production was 8.12±0.45 nmol/mgxmin) while longer exposures at much lower concentrations (2.3 μM thioacetamide) produced significant inhibition: 25% after 48h and 28% after 72 h. Control urea production was 9.30±0.92 and 9.52±0.5 nmol/mgxmim at the same times (Fig. 6).

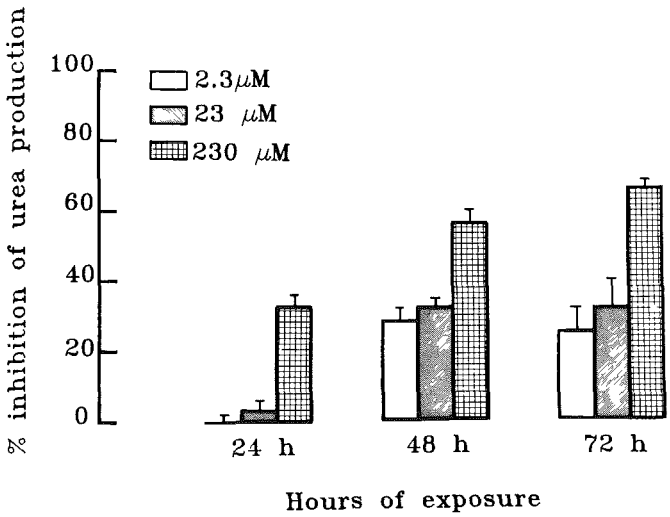

Fig. 6. Inhibitory effect of thioacetamide on urea production by hepatocytes. 24-Hour cultures were incubated with varying concentrations of the compound for 24 hours, 48 hours and 72 hours. The urea production rate was then assayed in the culture medium during 120 min after a load with 4 mM ammonium chloride.

The response of hepatocytes to drug enzyme inducers was assessed by incubating hepatocytes with phenobarbital and 3-methyl cholanthrene. AHH is a microsomal enzyme that is induced by 3-methyl cholanthrene "in vivo" but not by phenobarbital (38). ETC, on the other hand, belongs to a group of enzymes inducible by phenobarbital but not by 3-methyl cholanthrene. After 48 h exposure of hepatocytes to 1.85 μM 3-methyl cholanthrene, the AHH activity increased by 168 % and the ETC activity increased by 277% as compared with controls. Upon exposure

for the same time to 2 mM phenobarbital, the AHH activity was not significantly induced (6.27±1.01 versus 6.03±0.91 pmol/mg x min) while the ETC activity increased by 238% over that of the control (Table III). AHH was induced by 3-methyl cholantrene and ETC was induced by both inducers at concentrations and exposure times like those previosly reported (6,16).

TABLE III
MICROSOMAL ENZYME INDUCTION IN CULTURED HEPATOCYTES

Treatment	AHH activity (pmol/mg x min)	ETC activity (pmol/mg x min)
3-Methyl-cholanthrene	17.1 ± 2	81.9 ± 9.9
Phenobarbital	6.3 ± 0.01	72.8 ± 12
Control	6.4 ± 0.9	21.7 ± 2.9

The results presented above and others already reported by us (17-19) are in agreement with those of other authors in that they show that cultured hepatocytes can be used to understand the effects of xenobiotics on liver (4-9). Yet by using of metabolic parameters instead of just cytotoxic indexes, a more accurate picture of the effects of a potential hepatotoxin on liver cells is achieved.

A key point, however, is the practical extrapolation of results obtained "in vitro" to those to be expected "in vivo". A first aspect of this problem is the sensitivity of the parameters proposed and, in connection with this, the question of how sensitive the "in vitro" system is. The second aspect of the problem is how predictive the model can be. To be of practical value, an "in vitro" system must be sensitive enough to detect all potential hepatotoxins tested without false negatives and false positives. The choice of the parameters for "in vitro" toxicity and the nature of the culture influence the sensitivity of the biological assay. In our experimental approach, the question was formulated in another way. How sensitive is the system in detecting the effects of well-known hepatotoxins?. In other words, would our biological system be able to detect the hepatotoxicity of a compound in a blind test?. At what concentration in relation to the "in vivo" toxicity would a hepatotoxin be detected by the assay?, that is to say, how do the "in vitro" toxic concentrations correlate with the "in vivo" observed toxic doses.

In an attempt to answer these questions, the four toxics were handled as if they were unknown substances and the whole screening protocol was applied to each of them. Then the most sensitive parameter for detecting the toxic effects of each compound was tabulated together with the lowest concentration at which the compound produces effects on hepatocytes (Table IV). In addition, representative values taken from literature for "in vivo" toxic concentrations of the four compounds were considered. From this data and taking into consideration the pharmacokinetics of each compound, we estimated the "in vitro" equivalence of the "in vivo" toxic concentration and compared the limit of detection of the biological assay with the "in vivo" data. In the case of a-amanitin, a toxic that is largely taken up by the liver (32,33), it seemed reasonable to calculate the toxicity on the basis of liver weight/cell weight.

TABLE IV
A COMPARISON OF THE "IN VITRO" AND "IN VIVO" TOXICITY OF INDIRECT HEPATOTOXINS

Hepatotoxin	"In vivo" Toxicity	Estimated "in vitro" equivalence of toxicity	Concentration at which "in vitro" toxicity was first observed	Most sensible parameter
α- Amanitin	50 μg/100 g bw[33]	10 ng/mg of cel prot (a)	1.5 pg/mg of	RNA synthesis Albumin synthesis
D- Galactosamine	200 mg/Kg bw[35] 375 mg/Kg bw[34]	1.85-3.5 mM (b)	40 μM	LDH leakage
Thioacetamide	50 mg/kg bw[36] 200 mg/Kg bw[37]	1.33-5.32 mM (b)	2.3 μM	Ureogenesis
Acetaminophen	250 mg/Kg bw[30] 500 mg/Kg bw[30] 1500 mg/Kg bw[31]	1.74-10.44 mM (c)	0.1 mM	LDH leakage

(a) calculated on the basis of liver weight/liver cells weight.
(b) Data from the literature, assuming a distribution volume of 50 ml/100g bw.
(c) Data from the literature, assuming a distribution volume of 95 ml/100g bw.
Numbers above data of "in vivo" Toxicity correspond to literature references.

A lethal dosis of 50 μg/100 body weight (32,33) would then be equivalent to 10 ng/mg hepatocytes, assuming an average liver weight of 10 g. D-galactosamine and thioacetamide are water soluble drugs, and their distribution volumes are close to 50 ml/100 g b.w. (39) The toxicity of acetaminophen was calculated assuming a distribution volume of 95 ml/100 g b.w. (40). The data obtained in our study shows that toxic effects were clearly observed at concentrations lower than those expected from theoretical calculations, which speaks in favour of the sensitivity of this biological assay.

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Discussion - The use of cultured hepatocytes to assess the hepatotoxicity of xenobiotics.

P. Moldeus

A general limitation of cultured hepatocytes is the rapid loss of enzyme activity. Decreased cytochrome P 450 activity, for instance, would lead to lesser metabolic activation. Co-culturing with endothelial cells has been suggested to overcome these problems.

J.V. Castell

Yes, it is easy to get cells surviving, but what we aim to, and achieve, is a system that not only does look like a liver cell culture but that actually behaves like liver cells, with all their functions intact. We are careful with the timing of our experiments in order to avoid loss of enzyme activities. P 450 activity can be prolonged to some extent by the addition of suitable hormones and, of course, in special cases when biotransformation capability needs to be sustained over a long period, one can resort to co-culturing.

T. Wieland

Did you examine phalloidin in your system?

J.V. Castell

Phalloidin inhibits the extension of the cells, the spreading that leads to the development of the monolayer. This is a toxic effect on proteins in the cytoskeleton responsible for the flattening of the cells.

L.F. Prescott

What is the protein concentration in the fluid which mantains the cells? Many drugs are bound to plasma proteins and the amount of free drug in the solution could vary depending on that.

J.V. Castell

In many cases this fluid contains between 10 and 20% fetal calf serum. However, cultures can be achieved in serum-free medium, and the results reported here correspond to this type of culture. When we test drugs, we take into account protein binding and use concentrations equivalent to those of the free drug in the plasma.