MAINTENANCE OF CYTOCHROME P-450 AND METABOLISM OF AFLATOXIN B_1 IN PRIMARY HEPATOCYTE CULTURES

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SUMMARY: The cytochrome P-450 content of primary hepatocyte cultures was maintained at levels close to those found in vivo by using a defined medium containing testosterone, thyroxine, hydrocortisone, estradiol, glucagon, insulin, linoleic acid and oleic acid. Using these cultures, [^{14}C]aflatoxin B_1, a potent liver carcinogen, was metabolized primarily to water-soluble metabolites. In agreement with in vivo results, aflatoxin M_1 was the only nonpolar metabolite detected. In addition, a significant portion of radioactivity was covalently bound to cell constituents. These results suggest that primary hepatocyte cultures may be a good model of the liver for studying the metabolism and mechanism of action of toxic chemicals.

INTRODUCTION

Recently, primary hepatocyte cultures have been developed, providing intact cells which better reflect hepatocytes in vivo (1,2,3). Some workers have also used primary hepatocyte cultures to study the induction of drug-metabolizing enzymes (4,5) and the metabolism of toxic chemicals (6,7). However, the cytochrome P-450 mixed-function oxidase, probably the most important enzyme system for metabolizing toxic chemicals, was present only at low levels (2,3) and therefore these cultures were not useful for metabolic studies. Previous workers have suggested that hormones are required for maintaining the cytochrome P-450 content in liver (8). To establish primary hepatocyte cultures which would reflect the metabolic capability of hepatocytes in vivo, several hormones were used to maintain the level of cytochrome P-450 in culture. Then the metabolism of a potent liver carcinogen, aflatoxin B_1, was studied to determine whether the data obtained from these cultures could be extrapolated to the in vivo state.

MATERIALS AND METHODS

Isolation and culturing of hepatocytes. Hepatocytes were isolated from 180-210 gm Sprague-Dawley male rats (9). Each liver yielded 600-700 x 10^6 hepatocytes having greater than 95% viability as determined by trypan blue

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exclusion. Hepatocytes were suspended in Waymouth's medium MB752/1 (Gibco), modified as described below, and 20 ml containing 10^6 cells/ml were plated on 150 x 15 mm plastic petri dishes (Lab-Tek) pre-coated with rat tail collagen. Each plate contained 0.6 mg rat tail collagen as determined by the Lowry method (10) using calf skin collagen (Type III, Sigma) as a standard. The procedure was completed within one hour. Incubation was at 37C in a humidified 5% CO_2-95% air incubator. The medium was changed after 4 hr, 24 hr and every 24 hrs thereafter, unless otherwise noted.

Chemicals. Ring-labeled [^{14}C]aflatoxin B_1 and unlabeled metabolites of aflatoxin (aflatoxicol, aflatoxins Q_1, M_1, H_1, P_1, and B_{2a}), were prepared and their purity was confirmed as previously described (11). All biochemicals, unless otherwise noted, were from Sigma Chemical Co. All solvents were nanograde.

Culture media. Hepatocytes were cultured in Medium A or Medium AB as described below. Medium A contained per liter: Waymouth's medium MB752/1 (Gibco), 2 gm bovine serum albumin (fraction V, fatty acid poor, Miles Laboratories), 5 mg oleic acid, 5 mg linoleic acid, 11.2 mg L-alanine, 12.8 mg L-serine, 24 mg L-asparagine, 8 mg insulin (bovine pancreas, crystalline), 100 mg streptomycin sulfate, 100 mg gentamicin sulfate (Gentocin^R, Schering Corp., N.J.), 2.24 gm $NaHCO_3$; the medium was equilibrated with 5% CO_2-95% air and the pH adjusted to 7.4. Medium A was modified to medium AB by adding δ-aminolevulinic acid (10^{-6} M), DL-α-tocopherol acetate (5 mg/liter), D-thyroxine (10^{-5} M), testosterone (10^{-6} M), glucagon (5.07×10^{-8} M), hydrocortisone-21-acetate (10^{-5} M) and β-17-estradiol (10^{-6} M). The latter six compounds were added to the medium in propylene glycol. Propylene glycol never exceeded 0.38% of the media. All media were filter sterilized (0.22 μm, Type GS, Millipore Corp.).

Cytochrome P-450. The cytochrome P-450 content of hepatocytes was determined immediately after isolation. A portion of the hepatocytes (approximately 7×10^7 cells) was washed once with 30 ml of ice-cold phosphate-buffered saline (Ca^{2+} omitted) (12) by centrifugation at 50 x g for 5 min. After resuspension in 5.0 ml of the same buffer, the cells were homogenized with a tight-fitting teflon pestle (20 strokes) and two 0.2 ml portions of the homogenate were saved for DNA determination (13). The remainder was centrifuged at 10,800 x g for 15 min and the cytochrome P-450 concentration of the supernate was determined using a Cary 15 spectrophotometer, assuming an extinction coefficient of E_{450}-E_{490} nm = 91 cm^{-1} mM^{-1} (14). In cultured hepatocytes the cytochrome P-450 content was determined as above except for the following: after 20-24 hr in culture the medium was aspirated (15-16 x 10^6 cells/plate, 3-5 plates per assay) and the surface washed by aspiration two times with 10 ml of phosphate-buffered saline (Ca^{2+} omitted). Hepatocytes were scraped from the plate with a rubber spatula, suspended in the same buffer and the cytochrome P-450 and DNA concentrations determined as described above. Cytochrome P-450 content was expressed as pmoles P-450 per μg DNA.

DNA content of hepatocytes. DNA was determined in freshly isolated cells or in cultured cells after trypsinization from the plates (12). The cells were washed in 30 ml of ice-cold phosphate-buffer saline (Ca^{2+} omitted) by centrifugation at 50 x g for 5 min, counted in a Petroff-Hauser counting chamber and the DNA concentration of the cell suspension was determined colorimetrically (13).

Incubation of hepatocyte cultures with aflatoxin B_1 and analysis of metabolites. After 21 hr in culture with Medium AB, 20 ml of fresh Medium AB containing [^{14}C]aflatoxin B_1 (0.25-0.33 μCi/4.9-6.6 μg) in 0.01 ml propylene glycol was added to cultured hepatocytes (15 x 10^6 cells/plate). Control plates contained the same medium without cells. Incubation was at 37C in 5% CO_2-95% air. Immediately after adding fresh medium plus [^{14}C]aflatoxin B_1 and at 1,2, 4,6, and 10 hr thereafter, 1.0 ml of the medium was removed from each plate and added to 1.0 ml of ice-cold methanol. This mixture was extracted 3 times with 2 ml of $CHCl_3$. The $CHCl_3$ phase was washed with 2.0 ml of distilled water and the $CHCl_3$ extract was then analyzed for all known metabolites of aflatoxin B_1 by thin-layer chromatography as described previously (11). After 10 hr of incubation, the remaining medium was aspirated from the plates and the surface

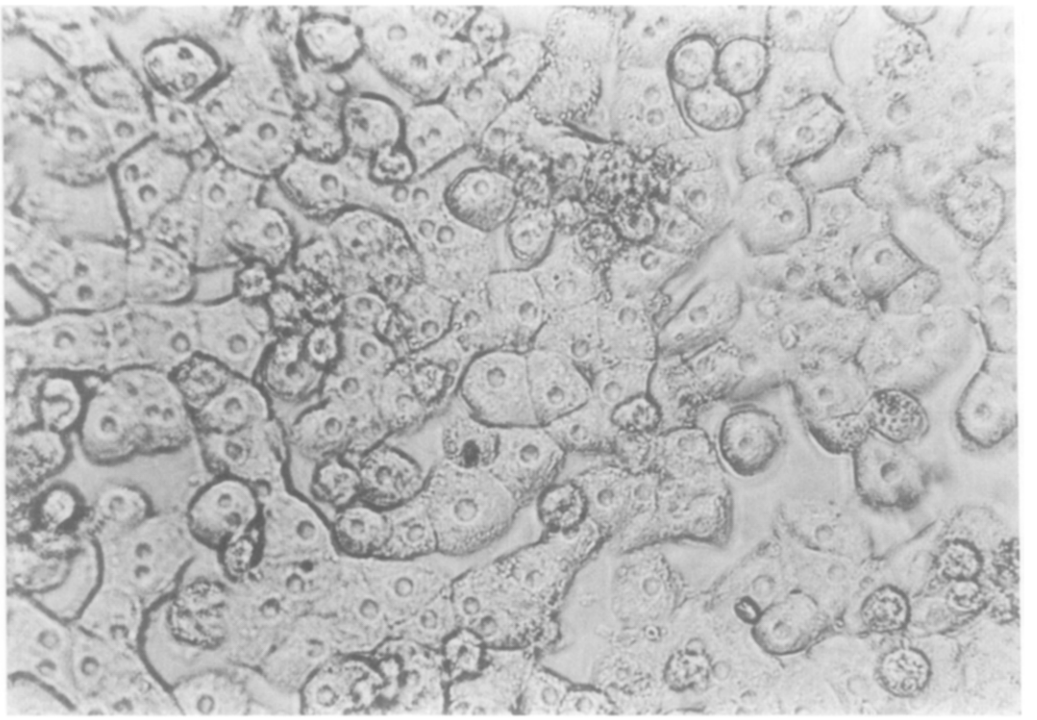

Fig. 1. Rat hepatocytes cultured in Medium AB for 21 hr.

washed twice with 20 ml of ice-cold phosphate-buffered saline (Ca^{2+} omitted). Cells were scraped from the plates with a rubber spatula, resuspended in the same buffer, homogenized (20 strokes), and centrifuged at 10,800 x g for 15 min. The 10,800 x g pellet was saved and the supernate was centrifuged at 100,000 x g for 1 hr. The 100,000 x g supernate, the microsomal pellet and the 10,800 x g pellet were extracted with $CHCl_3$ as described above. The extracted pellets were collected on glass fiber filters, washed 10 x with 2 ml $CHCl_3$, the filters were transferred to a count vial, and 1 ml Protosol (New England Nuclear Corp.) was added to each vial. After heating at 70 C for 1 hr, 10 ml Handifluor was added (New England Nuclear Corp.). Ten ml of Handifluor were also added to portions of aqueous and $CHCl_3$ extracts and radioactivity was determined with a Packard Tri-Carb Model 2425 liquid scintillation spectrometer. [^{14}C]Toluene (New England Nuclear) was used as an internal standard for quench correction.

RESULTS

Cytochrome P-450. In order to study the metabolism of foreign chemicals in cultured hepatocytes, the cytochrome P-450 content had to be maintained at levels close to those found *in vivo*. Therefore, hormones which were known to increase cytochrome P-450 *in vivo* (8) were added to a defined culture medium. The cytochrome P-450 content was determined in fresh hepatocytes and after 21-24 hr in culture. This length of time in culture was sufficient for the

TABLE 1. Cytochrome P-450 Content of Hepatocytes

	Cytochrome P-450 (pmoles) (μg DNA)		No. expts.	Cytochrome P-450 (% Fresh cells)
	Mean	Range		
Fresh cells	7.2	6.9-7.4	(8)	100
Cultured cells				
Medium A	2.7	2.3-3.8	(8)	38
Medium A + testosterone	4.0	3.8-4.2	(2)	56
Medium AB minus estradiol	5.5	4.7-6.2	(4)	76
Medium AB	7.2	5.4-8.4	(4)	100

See methods for the composition of media and determination of cytochrome P-450 and DNA.

formation of monolayers (Fig. 1); these cultures were also suitable for metaboli studies since any membrane damage incurred during the isolation procedure was repaired (9). Table 1 shows the effects of various hormone-supplemented media on the cytochrome P-450 content of cultured hepatocytes. In Medium A the cytochrome P-450 content was 38% of the level found in freshly isolated hepatocytes, in close agreement with previous workers (2,3). However, in Medium A plus 10^{-6} M testosterone, the cytochrome P-450 content increased to 56% of freshly isolated hepatocytes. Propylene glycol had no measurable effect on cytochrome P-450 content (not shown). Cytochrome P-450 was quantitated per pg DNA since the DNA content of the nondividing hepatocyte culture did not change over the culture period (12.0 pg DNA/cell).

The cytochrome P-450 content of hepatocytes cultured in Medium AB was close to the level found in freshly isolated hepatocytes (Table 1). Figure 2 shows the cytochrome P-450 spectra in an experiment with fresh and cultured hepatocytes from which some of the data in Table 1 were derived. Cells cultured in Medium A, Medium AB minus estradiol, or Medium AB, contained 50, 70, and 104% respectively of the cytochrome P-450 content of fresh hepatocytes. Since the level of cytochrome P-450 in hepatocytes cultured in Medium AB was close to that

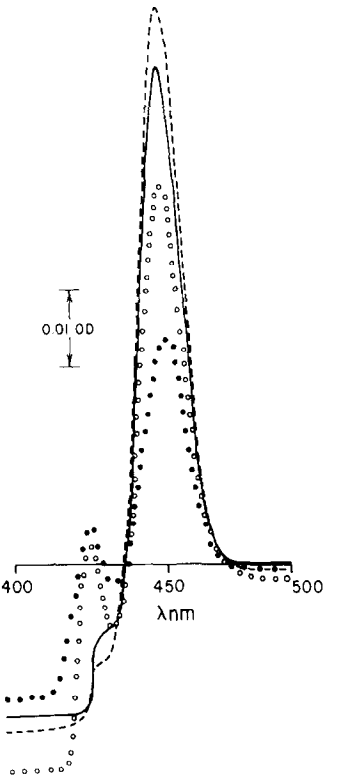

Fig. 2. Reduced CO difference spectra of 10,800 x g supernates from freshly isolated and cultured hepatocytes. Fresh hepatocytes (——); hepatocytes cultured for 21 hr in Medium A (●), Medium AB minus estradiol (o), or Medium AB (– –).

found in fresh hepatocytes, these cultures were used to study the metabolism of aflatoxin B_1.

Metabolism of aflatoxin B_1. The ability of the cultures to metabolize [^{14}C]aflatoxin B_1 was tested by analyzing the culture medium for water-soluble and $CHCl_3$-extractable metabolites. The concentration of water-soluble metabolit reached a plateau at 4 hr and remained high for up to 10 hr (Fig. 3A). This water-soluble radioactivity probably represents conjugates of aflatoxin (15). In addition, aflatoxin M_1 was the only $CHCl_3$-extractable metabolite observed by thin-layer chromatography (Fig. 3B). Table 2 shows that the major portion of the input radioactivity (57%) was recovered in the aqueous phase of the culture

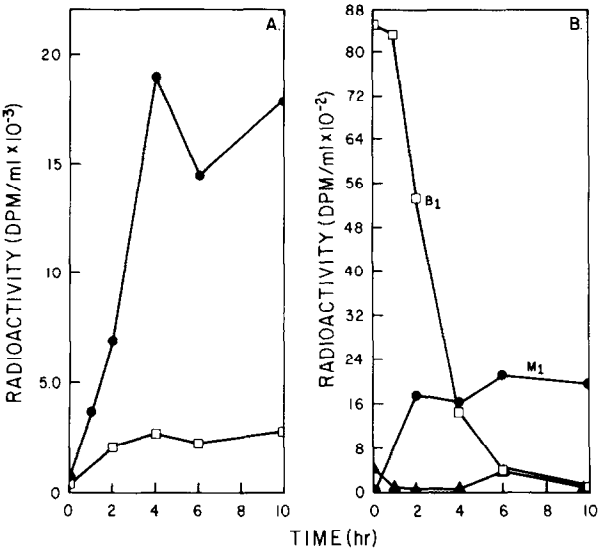

Fig. 3. Time course of [^{14}C]aflatoxin B_1 metabolism in cultured hepatocytes. A representative experiment is shown. A. Radioactivity recovered in aqueous phase of $CHCl_3$-extracted culture medium. [^{14}C]Aflatoxin B_1 was incubated in Medium AB with (●) or without (□) cultured hepatocytes. B. Resolution by thin layer chromatography of $CHCl_3$-extractable radioactivity recovered from culture medium. (□) Aflatoxin B_1; (●) aflatoxin M_1; (▲) radioactivity remaining at origin.

medium; only 5.3% of the input was $CHCl_3$-extractable (0.3% as aflatoxin B_1 and 5.0% as aflatoxin M_1) at 10 hr.

Binding of [^{14}C]aflatoxin. Radioactivity bound to the hepatocytes was determined after 10 hrs in culture. As shown in Table 2, 12.4% of the input radioactivity was $CHCl_3$-nonextractable and presumably covalently bound to various subcellular fractions.

DISCUSSION

Mixed-function oxidases are probably the most important enzyme systems for metabolism of foreign chemicals (8). Therefore, to establish a cell model for liver metabolism of foreign chemicals, it was essential to maintain the mixed-function oxidases at *in vivo* levels. Although previous workers showed that many biochemical functions of liver cells are retained in primary hepatocyte cultures (1,2,3), an important component of the mixed function oxidases,

TABLE 2. Recovery of [^{14}C]Radioactivity from Hepatocyte Culture Incubated with [^{14}C]Aflatoxin B_1

	$\frac{\text{Recovered } [^{14}C]^*}{\text{Input } [^{14}C]} \times 100$
Water-soluble metabolites	57.2
$CHCl_3$-extractable metabolites (Aflatoxin B_1 and M_1)	12.2
10,800 x g pellet	
$CHCl_3$-nonextractable	7.4
$CHCl_3$-extractable	0.3
100,000 x g pellet	
$CHCl_3$-nonextractable	1.0
$CHCl_3$-extractable	0.2
100,000 x g supernatant	
$CHCl_3$-nonextractable	4.0
$CHCl_3$-extractable	0.4
Water-soluble	3.0
Wash buffer from culture dish	5.2
Backwash from $CHCl_3$ phases	1.4
Recovery	92.3

*Recovery of [^{14}C] radioactivity was determined as described in Methods. Representative data from one of four experiments are shown.

cytochrome P-450, declined rapidly with time. Before studying the metabolism of aflatoxin B_1 in primary hepatocyte culture, the level of cytochrome P-450 (and presumably other components of the mixed-function oxidases) had to be maintained. It has been shown that the addition of hydrocortisone to a medium containing 5% fetal calf serum (2), or δ-aminolevulinic acid to a defined medium (3) produced only slight increases in the cytochrome P-450 content of cultured hepatocytes. Therefore, a mixture of hormones known to affect the liver *in vivo* (8) was added to a defined culture medium and was found to maintain the cytochrome P-450 content close to the level found in fresh hepatocytes. The content of cytochrome P-450 in the fresh hepatocytes presumably reflects the *in vivo* level, since the cytochrome P-450 content was determined immediately after cell isolation. Work is in progress to determine the lowest effective concentration of each hormone and various hormone mixtures. In addition, the

cytochrome P-450 content of cultures incubated with hormones for time periods up to 10 days is being determined.

Hepatocyte cultures with *in vivo* levels of cytochrome P-450 completely metabolized aflatoxin B_1 in 10 hrs. Most metabolites were water-soluble. Presumably, these metabolites are conjugates of aflatoxin (15), and work is in progress to identify them. Only aflatoxin M_1 was observed in the $CHCl_3$-extractable phase. This is in contrast to *in vitro* microsomal metabolic studies where several metabolites were produced (11), but agrees with *in vivo* results where only aflatoxin M_1 has been observed (16). Thus, primary hepatocyte cultures, in contrast to *in vitro* microsomal studies, adequately reflect the *in vivo* metabolism of aflatoxin B_1. A significant portion of the radioactivity was nonextractable. Since the bound radioactivity remained after extensive washing with $CHCl_3$, this radioactivity most likely was covalently bound and agrees with *in vivo* results which have demonstrated the metabolic activation and covalent binding of aflatoxin (17). This covalently bound aflatoxin is probably the basis for its toxicity and carcinogenicity (16,17,18). Work is in progress to identify cellular components to which the radioactivity was bound. Thus, primary hepatocyte cultures, in a defined culture medium supplemented with hormones, appear to be a useful model of the liver for studying the metabolism and mechanism of action of environmental chemicals such as aflatoxin B_1.

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