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COMPARATIVE METABOLISM, CYTOTOXICITY, AND GENOTOXICITY OF CHEMICAL CARCINOGENS IN PRIMARY CULTURES OF HEPATOCYTES

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INTRODUCTION

A major research objective of toxicologists is to develop bioassays that are rapid, inexpensive, sensitive, and predictive of potency and mechanism-of-action in humans. Lifetime exposures to rodents are now the definitive bioassay for chemical carcinogens. These assays are expensive, take three years to complete, are usually not very sensitive, seldom predict potency in humans, and usually provide little information on mechanism-of-action. Since epithelial tissues are the major target of chemical carcinogens, there is a logical trend to develop new bioassays based on these tissues. Systems with differing levels of biological resolution, from the intact animal to cell free organelles, have proven useful. The intact animal offers the greatest integration of biological events to give responses most relevant to the live animal, but also has the highest variability and provides the least control over experimental conditions. Subcellular systems offer the least variability and greatest control, but are furthest removed from the live animal and therefore the interpretation of the response is often the most difficult.

Epithelial cells in culture offer an intermediate level of resolution that provides an intact cellular response to chemical carcinogens. Tissue, organ, and organism response are lost but the important integrated cellular responses of uptake, metabolism, mutagenesis, and altered phenotype can all be assessed in a highly uniform population of cells in a completely defined chemical milieu. Compared to cell suspensions, cultures of epithelial cells usually provide data that is more relevant to the in vivo state, since culturing permits repair of injury incurred from the isolation technique, reestablishment of cell-to-cell contacts, attachment to a collagen substratum for easy manipulation, and carrying out bioassays that require several days. Primary cultures are usually a better model of epithelial cells in vivo since they maintain their differentiated function better

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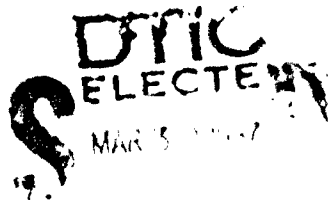

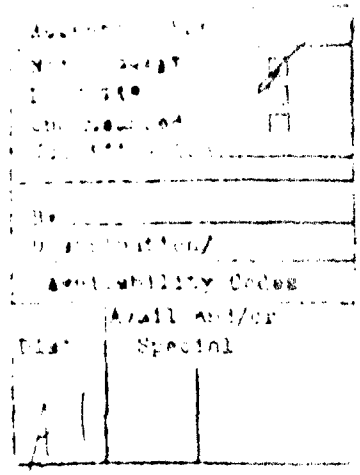

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than cell lines. Liver parenchymal cells, hepatocytes, have received considerable study due to their relative ease of isolation and the large number of a single cell type that can be prepared from a specimen of liver. The hepatocyte is also useful in the evaluation of chemical carcinogenesis for two reasons. The first reason is the high levels of mixed function oxygenase (MFO), an enzyme system critical for the activation of many procarcinogens. The second reason is the high level of DNA repair activity, providing a sensitive measure of chemical injury to DNA, the presumed first step in the initiation of carcinogenesis. The studies of chemical carcinogens in primary hepatocyte cultures reported herein are primarily those done in the author's laboratory.

MATERIALS AND METHODS

The techniques used in the author's laboratory for the isolation and culture of hepatocytes from adult mammals have evolved continuously over the past ten years (Dougherty et al., 1980; Decad et al., 1977; Green et al., 1981; Reese and Byard, 1981). Perfusion of the liver in situ with collagenase (Howard et al., 1967; Berry and Friend, 1969; Seglen, 1976; Bonney et al., 1974; Bissell et al., 1973) is limited by cost of small laboratory mammals. The development of a biopsy perfusion technique (Reese and Byard, 1981) permitted the isolation of viable hepatocytes from any mammal, including humans. With this technique, bioassays can be carried out in human hepatocytes; also, comparative studies with rodents and humans permit the use of data generated at the cellular level to be used to facilitate the extrapolation of in vivo potency in lifetime bioassays in rodents to humans. Successful culturing of freshly isolated hepatocytes requires a preparation of high viability, usually in excess of 80% by the method of trypan blue exclusion. Sterile technique is crucial and is best achieved in a small controlled room with sterile laminar flow air. High density plating on a collagen substratum (Pariza et al., 1975) in an enriched tissue culture medium provides the greatest chance of obtaining a continuous monolayer that can be maintained for several days in a viable and differentiated state. Several hormones are added to the culture media to maintain critical differentiated functions, such as mixed function oxygenase (Decad et al., 1977; Dickins and Peterson, 1980).

Several measurements that are important to chemical carcinogenesis can be carried out in the hepatocytes. These measurements include cellular uptake, metabolism, covalent binding to

macromolecules, induction of enzymes, cytotoxicity, and stimulation of DNA repair. Uptake, metabolism and covalent binding are most easily assessed with a radiolabeled carcinogen. For organic carcinogens of low water solubility, extraction of the culture media with an organic solvent provides a means of separating the parent compound from water soluble metabolites (Decad et al., 1977; Spilman and Byard, 1981; Green et al., 1981; Steward and Byard, 1981). When measuring the cytotoxicity and/or genotoxicity of a chemical, a measure of the metabolism of a procarcinogen is essential to be sure that all of the parent compound has been metabolized and therefore all of the potential for formation of toxic metabolites has been realized within the time of the bioassay. The culture media can be easily removed from the monolayer to permit measure of cellular uptake and covalent binding to cellular macromolecules (Decad et al., 1977; Spilman and Byard, 1981; Green et al., 1981; Salocks et al., 1981; Loury and Byard, 1984; Salocks et al., 1984).

Induction of enzymes is important in chemical carcinogenesis because many chemicals act as cocarcinogens or as anticarcinogens by increasing enzymes for activation or inactivation of initiators. Induction of MFO in primary hepatocyte cultures can be assessed directly by incubation with the inducer followed by enzymatic or spectrophotometric measure of MFO (Michalopoulos et al., 1976; Dougherty et al., 1980; Steward and Byard, 1981).

Cytotoxicity is an important measurement for chemical carcinogens because this measure places an upper bound on bioassays for genotoxicity and indicates the potential for promotion by means of cell death and regeneration. Cytotoxicity is easily assessed in primary hepatocyte cultures by light microscopic observation (Green et al., 1981) and/or the leakage of soluble cytosolic enzymes (Anuforo et al., 1978; Green et al., 1981; Salocks et al., 1981).

Genotoxicity can be assessed in primary hepatocyte cultures by chemical injury to DNA, measured either as covalently bound adducts (Salocks et al., 1984; Loury et al., 1984) or the stimulation of DNA repair to remove these adducts (Williams, 1976; Probst et al., 1981; Green et al., 1981; Loury and Byard, 1983). The assay for the stimulation of DNA repair is done by measuring the uptake of [H-3]thymidine into DNA. One technique involves autoradiography of the culture following incubation with chemical carcinogen and [H-3]thymidine (Williams, 1976). The advantage of this technique is the ability to visually distinguish replicating from repairing nuclei by the relative density of silver grains. The disadvantage is the approximately two

weeks required to carry out the assay. The recent development of a sensitive scintillation detection of repair provides a three day assay (Loury and Byard, 1983). A UV light positive control is used in this assay to normalize differences in individual animals, species, and other experimental parameters. Although culture conditions in this assay have been selected that minimize replication, and known stimulators of replication do not significantly increase replication in this system, a false positive due to stimulation of replication of the entire genome cannot be unequivocally ruled out. A significant positive finding in either the autoradiographic or scintillation assay of DNA repair should be followed-up by other measures of genotoxicity, such as adduct formation.

The primary hepatocyte culture technique provides a two-tiered biological system for assessing chemical-chemical interactions. Hepatocytes can be isolated from animals exposed to one chemical and then treated in vitro with a second chemical. Control animals provide a two-by-two protocol for assessing interaction. This technique has been used in this laboratory to assess the effect of butylated hydroxytoluene (Salocks et al., 1981; Salocks et al., 1984) and phenobarbital (Loury et al., 1984) on the toxicity of aflatoxin B-1, the interaction between PCB and amino acid pyrolysates (Loury and Byard, 1983), and the interaction between ethanol and amino acid pyrolysates (Loury and Byard, 1984).

RESULTS

The aromatic amine hepatocarcinogen, 2-acetylaminofluorene (2-AAF), was completely metabolized by primary rat hepatocyte cultures to water soluble metabolites and covalently bound adducts in 18 hours (Spilman and Byard, 1981; Figure 1).

A similar pattern was found for aflatoxin B-1 in hepatocyte cultures from mice and rats (Decad et al., 1979). The mouse produced more water soluble metabolites and about 20-fold less covalently bound adducts than the rat (Table 1).

In parallel with this finding, the rat is much more sensitive than the mouse to the carcinogenic effects of aflatoxin B-1. The extension of these data to rabbit and human (Salocks, 1983; Table 2) further emphasizes species variation in metabolism, and suggests that with the very limited data, humans are less susceptible than rats but considerably more susceptible than the mouse. Further replication of these data should provide a more certain measure of these apparent differences.

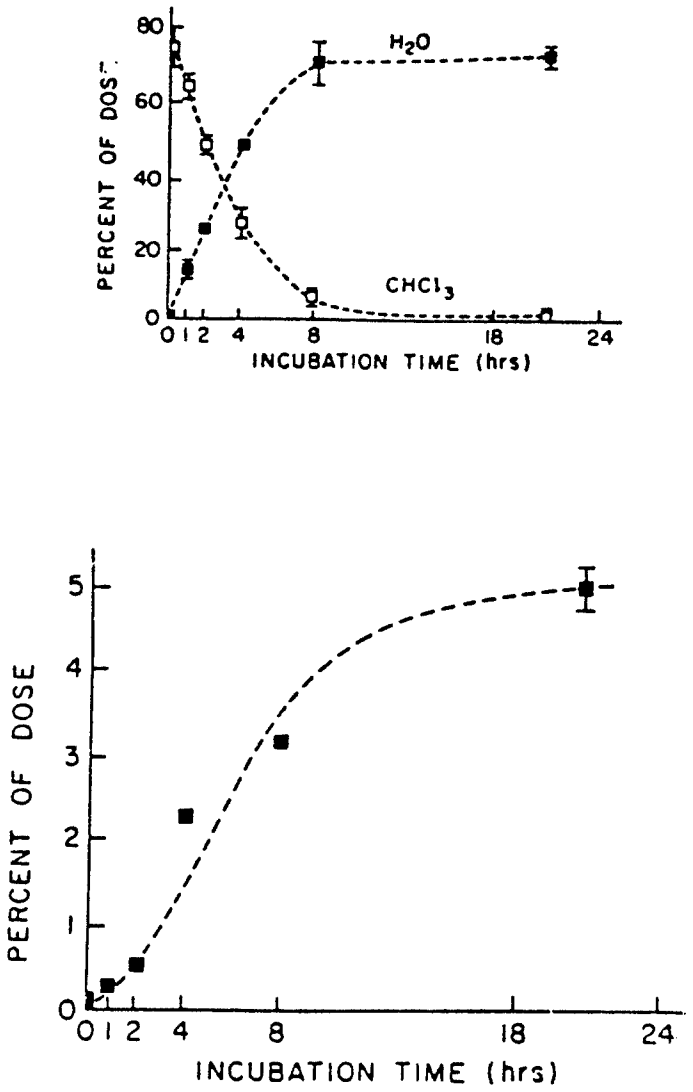

Figure 1. Metabolism of [14-C]AAF in rat hepatocyte cultures. Each culture received 137 ng of [14-C]AAF at 28 hours and metabolism was followed by an aqueous-chloroform partition of the culture medium. Covalent binding was measured by exhaustive washing of the trichloroacetic acid precipitate of the cells with aqueous and organic solvents. Published with permission of Hemisphere Publishing Company, Figures 2 and 4 in Spilman and Byard, 1981.

TABLE 1. METABOLISM OF [C-14] AFLATOXIN B-1 IN MOUSE AND RAT PRIMARY HEPATOCYTE CULTURES[a,b]

$$\frac{\text{Recovered [C-14]}}{\text{Input [C-14]}} \times 100$$

	Mouse	Rat
CULTURE MEDIUM		
Water-soluble metabolites	91.0	57.2
$CHCl_3$-extractable metabolites		
Aflatoxin B-1	0.0	0.7
Aflatoxin M-1	0.4	11.5
Wash from culture dishes	5.0	5.2
Aqueous wash from $CHCl_3$-extracts	1.0	1.4
HEPATOCYTES		
Covalently-bound radioactivity[c]	0.6	12.4
$CHCl_3$-extractable radioactivity	0.0	0.9
Water-soluble radioactivity	0.0	3.0
RECOVERY	97.9	92.3

[a] Freshly isolated hepatocytes were cultured for 21 hours and then incubated 10 hours with aflatoxin B-1.

[b] Published with permission from Academic Press; from Table 1 in Decad et al., 1977 and Table 1 in Decad et al., 1979.

[c] Covalently-bound radioactivity was determined by precipitating cellular macromolecules with trichloroacetic acid and exhaustively extracting the precipitate with $CHCl_3$.

TABLE 2. METABOLISM OF AFLATOXIN B-1 BY ADULT PRIMARY HEPATOCYTE CULTURES[a]

Species	N	t (hr)	Covalent binding at 10 hours (% of dose)
mouse	3	<1	0.8 ± 0.3
rabbit	1	5	7
rat	4	4	15.4 ± 2.6
human	1	11	5

[a] Rabbit and human data were by Salocks, Hsieh and Byard unpublished data. Rat was from Decad et al., 1977 and mouse data was from Decad et al., 1979.

Induction of MFO by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in primary hepatocyte cultures is an exquisitely sensitive bioassay for TCDD (Steward and Byard, 1981). This bioassay could be used to measure TCDD and its congeners in complex environmental samples. A measure of the species variation in MFO induction by TCDD can also be determined by comparative induction studies in primary hepatocyte cultures. For example, induction data in hepatocytes from rat, guinea pig, and human indicate striking species variation in response (Steward, 1982; Figure 2). The difference seen in induction between hepatocyte cultures from rats and guinea pigs has been measured in vivo (Poland and Glover, 1973; Hook et al., 1975).

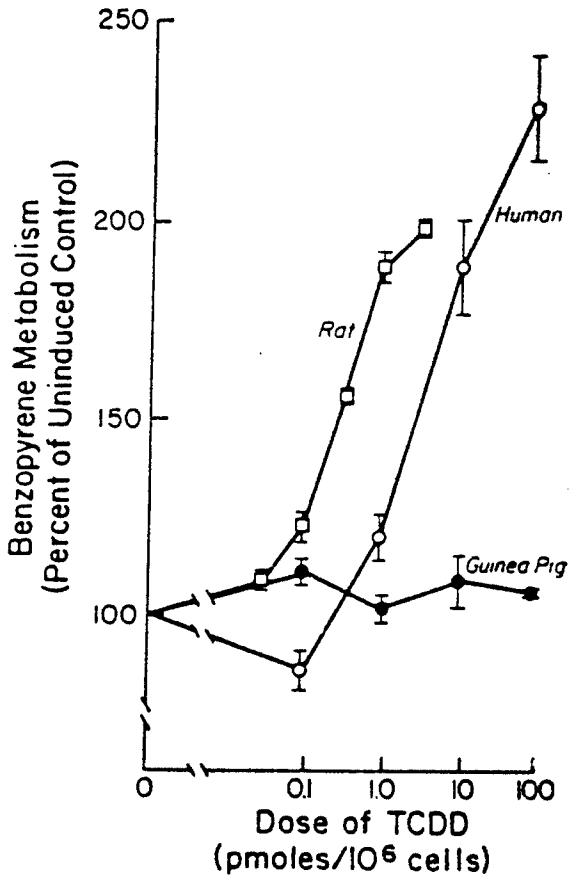

Figure 2. Comparative dose-response curves for induction by TCDD of primary hepatocyte cultures prepared from rat, guinea pig, and human liver. All points represent means ± SE (n = 4 cultures) from a single biopsy. TCDD was added to 6 hour cultures; benzo(a)pyrene metabolism was assessed 30-36 hours after the addition of TCDD.

A suspected hepatocarcinogen, senecionine, a pyrrolizidine alkaloid isolated from Senecio vulgaris, was both cytotoxic (Figure 3) and genotoxic (Figure 4) in primary hepatocyte cultures from rats (Green et al., 1981).

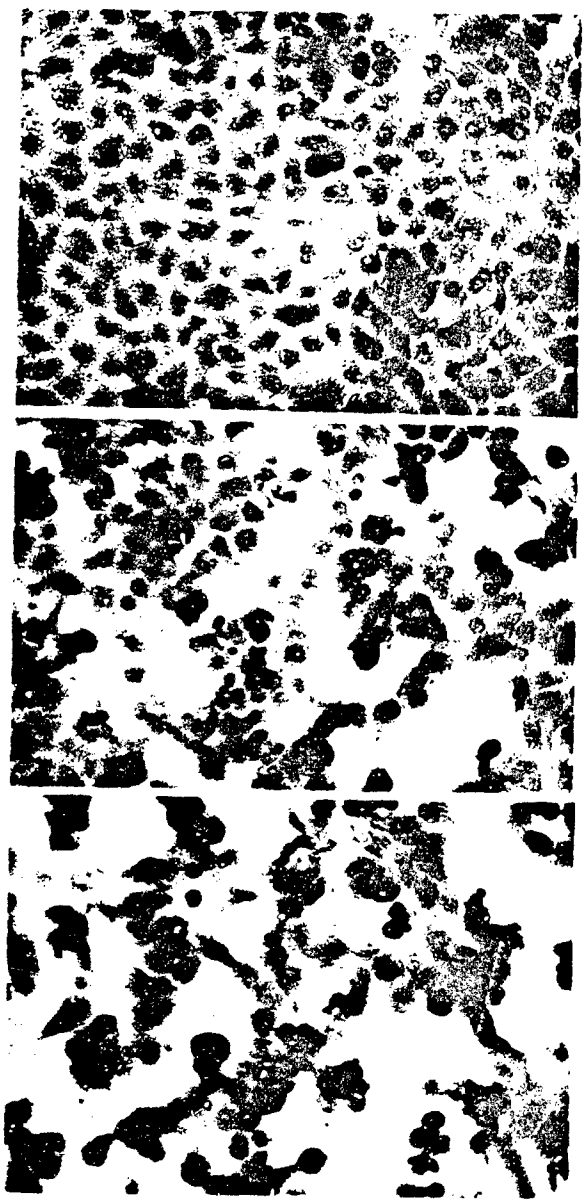

Figure 3. Photomicrographs of cultured rat hepatocytes after a 24 hour exposure to senecionine (200 X). Cells were fixed with glutaraldehyde and stained with hematoxylin. Top panel: control; middle panel: 80 nmol senecionine/million cells; bottom panel: 120 nmol senecionine/million cells. Published with permission of Academic Press; figure 3 in Green et al., 1981.

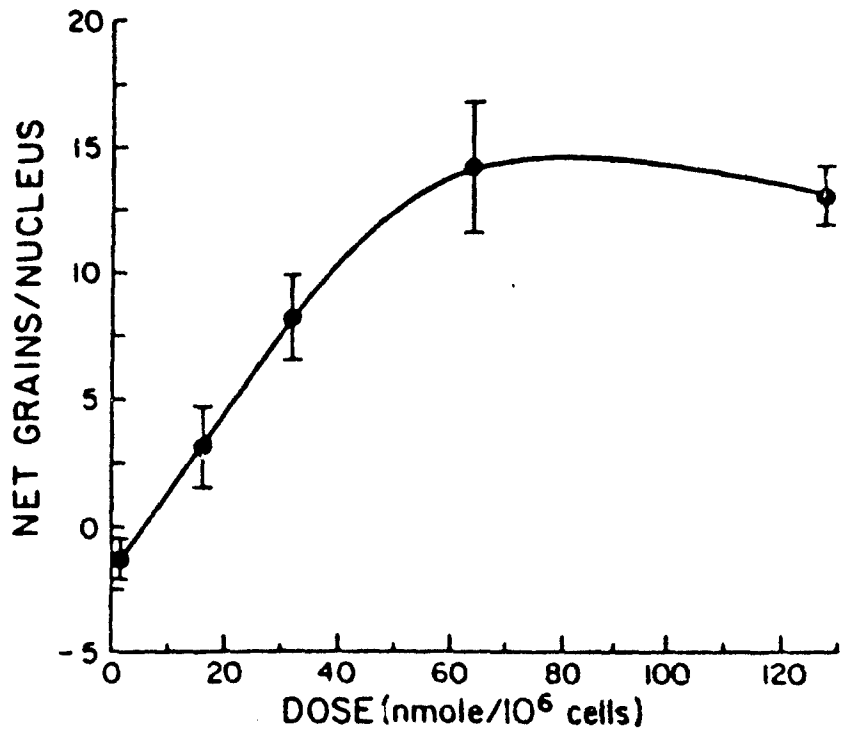

Figure 4. Stimulation of DNA repair by senecionine in primary cultures of hepatocytes. A representative experiment is shown (n = 3). Each value represents the mean ± SD of the average net grains/nucleus for three sets of 50 nuclei each. The control grain count was -0.8 ± 0.7. The grain count for the positive control, aflatoxin B-1 (1.5 nmol/million cells), was 39.3 ± 1.6. Published with permission of Academic Press; figure 4 in Green et al., 1981.

Based on these results, one would predict that senecionine would produce hepatic necrosis and hepatocarcinogenesis.

Butylated hydroxytoluene (BHT) is both an antioxidant and an inducer of MFO, epoxide hydrolase, and glutathione-S-transferase. Hepatocyte cultures from rats fed diets containing BHT metabolized aflatoxin B-1 slightly faster than control rats (Salocks et al., 1981). BHT hepatocytes yielded about 10% greater water-soluble metabolites and, very importantly, about a two-thirds reduction in covalently bound adducts (Figure 5).

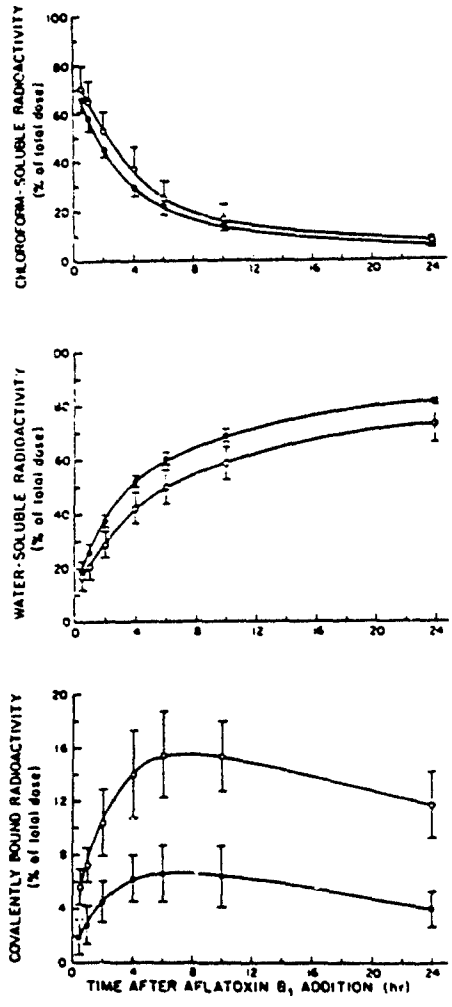

Figure 5. Effect of BHT pretreatment on metabolism of aflatoxin B-1 in primary cultures of hepatocytes. BHT hepatocytes were from rats fed 0.5% BHT for ten days. 120-150 ng [14-C]aflatoxin B-1 was added to 20 hour cultures of control (o) or BHT (o) hepatocytes. Data in the top two panels were from a chloroform extraction of culture media at the designated times after the addition of aflatoxin B-1. The covalent binding indicated in the bottom panel was the radioactivity remaining with the trichloroacetic acid extract of tne cells after exhaustive extraction with polar and nonpolar solvents. Three cultures from each of 3-4 rats per pretreatment group were analyzed at each time point. All points represent the means ± SD. Published with permission of Academic Press; Figures 3-5 in Salocks et al., 1981.

This reduction in biochemical injury correlated with protection by BHT against cytotoxicity as measured by leakage of lactic dehydrogenase (Figure 6).

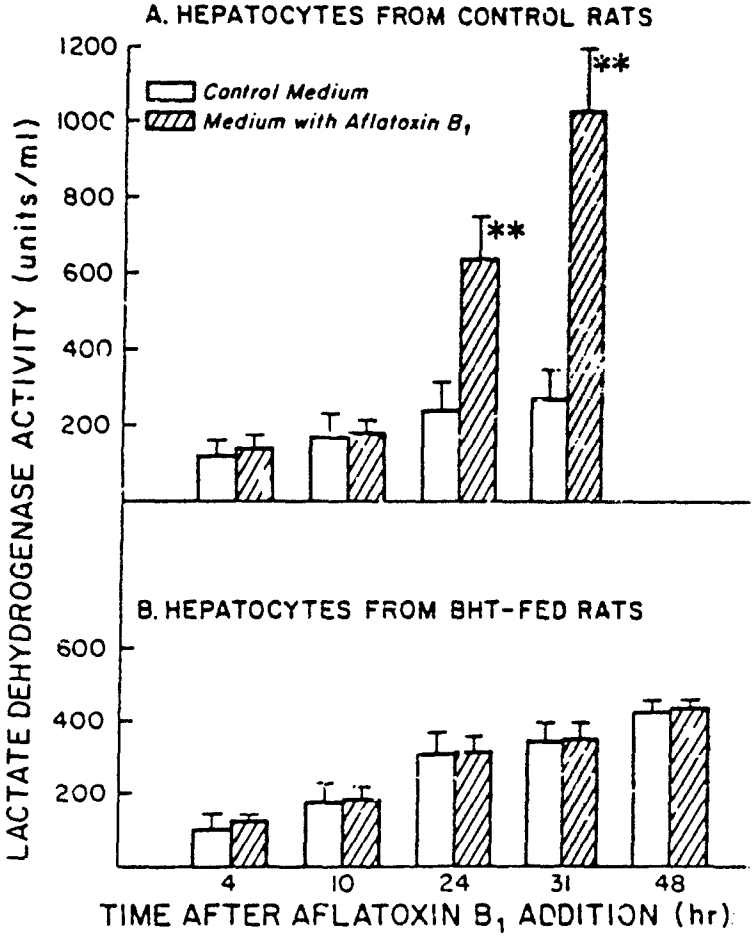

Figure 6. Effect of BHT pretreatment on the cytotoxicity of aflatoxin B-1 in primary cultures of adult rat hepatocytes. Cytotoxicity was assessed by measuring leakage of intracellular lactate dehydrogenase (LDH) into the culture medium of hepatocyte cultures from three rats fed a control diet (A) and three rats fed a diet containing 0.5% BHT (B). Values represent means ± SD. ** indicates values significantly higher than cultures without aflatoxin B-1 ($p < 0.01$, Student's t Test). Published with permission of Academic Press; figure 6 in Salocks et al., 1981.

Apparently, BHT preferentially induces enzymes that detoxify aflatoxin B-1. Separation of the cellular macromolecules into DNA, RNA, and protein revealed that DNA adducts were reduced 10-fold, RNA adducts 5-fold, with very little reduction in protein adducts (Salocks et al., 1984; Table 3).

TABLE 3. BINDING OF [^{14}C] AFLATOXIN B_1 TO MACROMOLECULES IN HEPATOCYTE CULTURES FROM CONTROL RATS AND RATS FED 0.5% BUTYLATED HYDROXYTOLUENE[a]

	Amount of macromolecule/culture[b]		pmole AFB_1 bound/mg macromolecule[c]		Ratio of bound products (BHT:control)
	Control	BHT-Pretreated	Control	BHT-Pretreated	
DNA	24.2 ± 2.1 µg	25.2 ± 1.6 µg	271 ± 53 (1.50)	25 ± 10 (0.14)[d]	0.092
RNA	231.0 ± 13.0 µg	282.8 ± 36.3 µg	341 ± 148 (7.61)	60 ± 11 (1.94)[d]	0.176
Protein	2.6 ± 0.3 mg	3.6 ± 0.1 mg	31 ± 5 (18.0)	22 ± 4 (14.9)[e]	0.71

[a] Each culture (2.5×10^6 hepatocytes) received 128 ng [^{14}C] AFB_1. Incubations lasted 6 hr. Values shown are means ± SD of 3 (control cultures) or 4 (BHT-pretreated cultures) determinations. Published with permission from Academic Press from Table 1 in Salocks et al., 1984.
[b] DNA, RNA and protein levels were determined in lysed sus pensions of equivalent cultures which had not undergone macromolecule fractionation.
[c] Numbers in parentheses indicate the % of the administered AFB_1 dose which was bound to the particular macromolecule species.
[d] Significantly less than control, $p < 0.01$ (Student's t-test).
[e] Significantly less than control, $p < 0.05$ (Student's t-test).

This result suggests that BHT will also protect against the hepatocarcinogenicity of aflatoxin B-1.

The development of a rapid scintillation measure of the stimulation of DNA repair permits the evaluation of genotoxic potency of a number of chemicals and the interaction of the genotoxic potency of chemicals with other chemicals. The assay conditions developed in this laboratory gave a sensitive detection of the hepatogenotoxic potency of aflatoxin B-1 (Loury and Byard, 1983; Table 4).

TABLE 4. THE STIMULATION OF UNSCHEDULED DNA SYNTHESIS IN PRIMARY HEPATOCYTE CULTURES AFTER TREATMENT WITH AFLATOXIN B-1[a]

Treatment	Concentration (M)[b]	dpm/ug DNA[c]	% Control
Aflatoxin B-1	none	142 ± 12	100
	1×10^{-9}	254 ± 7	178
	1×10^{-8}	608 ± 28	427
	1×10^{-7}	1749 ± 211	1130
	1×10^{-6}	2561 ± 177	1701

[a] Hepatocytes were isolated from adult male rats. Published with permission from Elsevier Scientific Publishers Ireland, Ltd.; from Table 1 in Loury and Byard, 1983.
[b] Total volume of culture medium was 4 ml.
[c] Values represent the means ± SD of quadruplicate samples.

The synergistic effect of PCB, given in vivo, on the genotoxicity of amino acid pyrolysates, added to primary hepatocyte cultures, illustrates the usefulness of this two-tiered system to detect cocarcinogens as well as anticarcinogens (Loury and Byard, 1983; Figure 7).

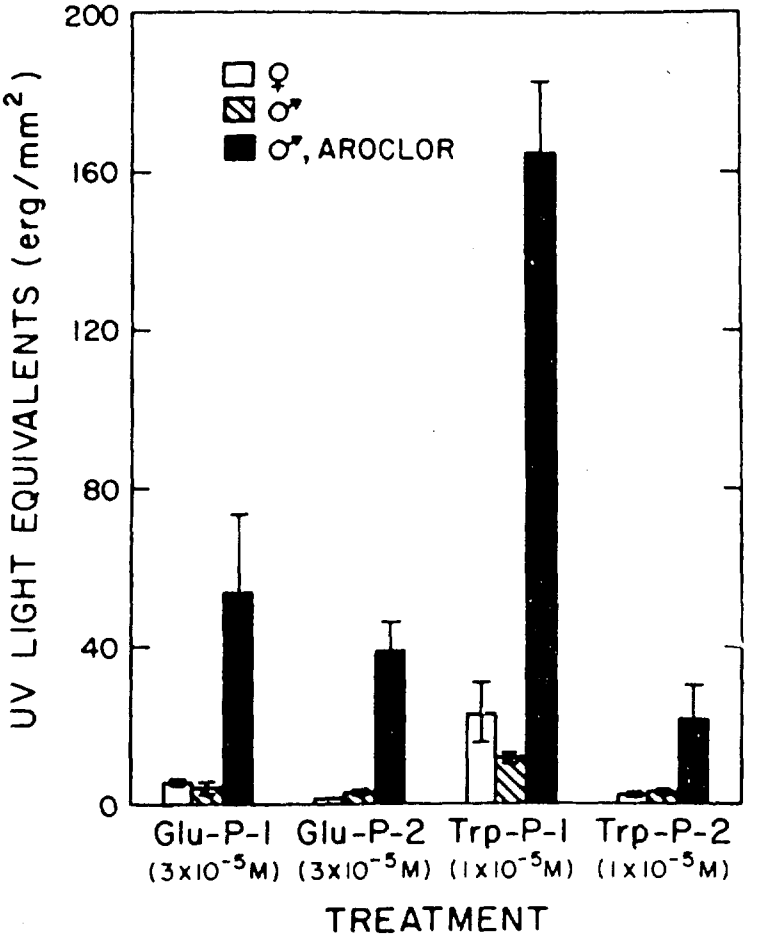

Figure 7. The stimulation of DNA repair by amino acid pyrolysates in primary hepatocyte cultures from control rats and rats pretreated with Aroclor 1254. DNA repair was measured in quadruplicate cultures from each rat and expressed in terms of UV light equivalents based on the DNA repair response of quadruplicate cultures exposed to 100 erg/square mm UV light. The background and response to UV light (dpm/ug DNA) of each group were: 137, 2229, male rats; 131, 1710, female rats; and 221, 1595, Aroclor-induced male rats. Values represent means ± SD (n = 3 rats/group). Published with permission of Elsevier Scientific Publishers Ireland Ltd., Figure 1 on p 287 of Loury and Byard, 1983.

DISCUSSION

The routine production of primary cultures of hepatocytes of high viability is technically demanding, but is being successfully done in several laboratories. The recent development of the biopsy perfusion technique has expanded application to human liver, enabling studies that will provide data to better predict human susceptibility to chemical carcinogens. Considerable work remains to be done in developing a completely defined culture medium that will best maintain hepatocytes in their fully differentiated state.

Although primary hepatocyte cultures provide a good cellular model of hepatocytes in vivo, they are often a poor model of the intact organism. Therefore, considerable caution must be used in interpreting data generated from primary hepatocyte cultures. An example is the finding that hepatocarcinogenic aromatic nitro compounds do not stimulate DNA repair in primary hepatocyte cultures (Probst et al., 1981). Apparently, the gut microflora are required to reduce the nitro group to the amine group which is then activated in the liver. Even with these limitations, for many carcinogens the quantitative assessment of metabolism and chemical injury to DNA, RNA, and protein in primary cultures of rodent and human hepatocytes, and the correlation of this chemical injury with cytotoxic and genotoxic endpoints, will provide comparative mechanistic data that can improve the assessment of human susceptibility.

ACKNOWLEDGEMENTS

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