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Inter-relationships among aflatoxin B_1 (AFB_1) metabolism, DNA-binding, cytotoxicity, and hepatocarcinogenesis in rainbow trout *Oncorhynchus mykiss*

Oliva Nunez*, Jerry D. Hendricks, Arthur T. Fong*****

Department of Food Science and Technology, Oregon State University, Corvallis 97331-6602, Oregon, USA

ABSTRACT: The roles of metabolism in aflatoxin B_1 (AFB_1) cytotoxicity and of cytotoxicity in AFB_1 hepatocarcinogenesis in rainbow trout *Oncorhynchus mykiss* have been examined. Groups of rainbow trout fry were exposed to carcinogenic aqueous solutions of 0.05, 0.1, 0.25, or 0.5 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Another group of fry was fed 500 mg l^{-1} β-naphthoflavone (BNF) for 1 wk before exposure to 0.5 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Subsamples of fish were killed 24 h and 2 wk later for DNA-binding and histopathological analysis, respectively. Results indicated a linear dose-response in both DNA-binding and cytotoxicity. BNF treatment resulted in a decrease in both DNA-binding and cytotoxicity. These results suggest that cytotoxicity, in common with carcinogenicity, is dependent on metabolism of AFB_1 to the electrophilic 8,9-epoxide that can react covalently with cellular macromolecules, and that cytotoxicity contributes to, but is not required for, hepatocarcinogenesis. In a separate experiment, groups of fry were exposed to 0 (control) or 0.5 mg l^{-1} AFB_1 for 30 min and subsamples of fish were given [^{3}H]-thymidine ([^{3}H]-TdR) i.p. at a single dose of 5 μCi g^{-1} body wt at 0, 1, 3, 7, and 14 d following carcinogen exposure, 24 h prior to necropsy. Autoradiograms showed intense radioactivity in presumptive oval cells which were seen at 14 d after carcinogen exposure, but no labeling in degenerate, necrotic hepatocytes. These results suggest that presumptive oval cells are responsible for liver regeneration.

INTRODUCTION

Aflatoxin B_1 (AFB_1), produced by certain strains of *Aspergillus flavus* and *A. parasiticus*, is a potent hepatotoxin and hepatocarcinogen in rainbow trout (Halver 1967, Bauer et al. 1969, Sinnhuber et al. 1977). It has been established that in the trout AFB_1 is metabolized by cytochrome P-450-dependent enzyme systems (Williams & Buhler 1983) to produce the electrophilic AFB_1-8,9-epoxide, and the covalent binding of AFB_1-8,9-epoxide to DNA has been proposed to be a critical event in the induction of AFB_1 carcinogenesis (Swenson et al. 1977).

In addition to AFB_1-8,9-epoxide, several other metabolites of AFB_1 including aflatoxicol (AFL), aflatoxin M_1 (AFM_1), and aflatoxicol M_1 (AFL-M_1) also are produced in control uninduced trout (Loveland et al. 1983). Glucuronides of AFL and AFL-M_1 are the major and minor biliary Phase II conjugates, respectively, in the untreated trout (Loveland et al. 1984). In contrast to mammalian systems in which glutathione (GSH) conjugation of the AFB_1-8,9-epoxide is a major detoxification pathway (Degen & Neumann 1978), GSH conjugation does not contribute significantly to the detoxification of AFB_1 in trout (Valsta et al. 1988).

Modification of AFB_1 metabolism and carcinogenesis in rainbow trout by several compounds including flavonoids, indoles, and polychlorinated biphenyls has been extensively studied in our laboratory (Bailey et al. 1982, 1984, Hendricks et al. 1982, Loveland et al. 1984, Nixon et al. 1984, Shelton et al. 1986, Dashwood et al. 1988). Pertinent to the objectives of this report are previous studies on the inhibitory effects of β-naphthoflavone (BNF) on AFB_1 carcinogenesis. Williams &

* Present address: Instituto Nacional de Investigaciones Agrarias, Embajadores 68, E-28012 Madrid, Spain
** Addressee for reprint requests
*** Present address: Environ, 5820 Shellmound St., Suite 700, Emeryville, California 94608, USA

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Buhler (1984) reported on the induction of the cytochrome P-450 isozyme, LM_{4b}, by BNF. The induction of cytochrome P-450 LM_{4b} results in a shift of AFB_1 metabolism away from the formation of AFL, a highly carcinogenic metabolite, to the formation of AFM_1 and AFL-M_1, metabolites of much less carcinogenic potential (Loveland et al. 1983). Significantly higher levels of AFL-M_1 glucuronide and reduced DNA binding by AFB_1 were observed in BNF-treated trout compared to control trout after AFB_1 exposure (Bailey et al. 1982, 1984, Loveland et al. 1984, Goeger et al. 1986). Therefore, the observed inhibition of AFB_1 carcinogenesis by BNF (Nixon et al. 1984, Goeger et al. 1986) appears to result from the altered metabolism of AFB_1 to less carcinogenic metabolites, increased conjugate formation, and reduced DNA binding.

Depending on the dose of AFB_1 administered, cytotoxicity often occurs as a concurrent lesion during experimental carcinogenesis. However, it is unclear whether or not cytotoxicity is causally related to carcinogenesis. Studies by Hoel et al. (1988) showed that cytotoxicity was not required for carcinogenesis but when it occurred, a promotional mechanism due to subsequent cell proliferation was postulated. Many hepatotoxins that cause cytotoxicity require metabolism to active metabolites which react covalently with tissue macromolecules (Zimmerman 1978). Nevertheless, the relationship between covalent DNA binding and cytotoxicity remains unclear.

In rainbow trout, the sequence of morphological events following exposure to chemical carcinogens and the biochemical bases for the morphological changes have not been systematically studied. In particular the relationships among carcinogen dose, DNA binding, cell specific cytotoxicity, regeneration, and the development of neoplasia have not been defined. In this report we have focused on the initial 2 wk following AFB_1 exposure in order to define the inter-relationships among dose, DNA-binding, target cells, cytotoxicity, and early regeneration.

In addition, autoradiography was used after i.p. administration of [^{3}H]-TdR, which is incorporated exclusively into DNA (Hughes et al. 1958), in order to evaluate which cell population was undergoing proliferation in the early cytotoxic/regenerative step of hepatocarcinogenesis.

MATERIALS AND METHODS

Experiment 1. ***Protocol:*** Groups of 55 fry (3 wk after swim-up) reared at the Food Toxicology and Nutrition Laboratory, Oregon State University, USA, were exposed to static aqueous solutions of carcinogenic doses of 0.05, 0.1, 0.25, or 0.5 mg l^{-1} [^{3}H]-AFB_1 (250 µCi l^{-1} H_2O) for 30 min. A separate group of fry was fed 500 mg l^{-1} BNF for 1 wk before exposure to 0.5 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Following carcinogen exposure, the fish were transferred to fresh water without AFB_1 and triplicate groups of 15 fish each were killed 24 h after carcinogen exposure. Livers were removed, pooled, and frozen in liquid N_2 before storing at −70 °C.

Isolation of DNA: DNA was isolated from groups of 15 pooled livers using a chloroform-phenol extraction method (Dashwood et al. 1988).

The DNA was precipitated with 160 µl 4 M sodium acetate (NaOAC) and 3.2 ml ice-cold ethanol, and collected by centrifugation (5000 × *g*, 5 min). The precipitate was washed 3 times with ethanol to remove unbound AFB_1, and dried under a gentle stream of N_2. The DNA was dissolved in 1.0 ml Tris-buffer, an aliquot of which was taken for DNA determination by the fluorometric method of Cesarone et al. (1979) and a further aliquot was assayed for radioactivity by liquid scintillation counting.

Data from [^{3}H]-AFB_1-DNA binding were analyzed by weighed linear regression analysis (Steel & Torrie 1980).

Histopathology: Groups of 10 fish were sampled 2 wk after carcinogen exposure. Livers were removed and fixed in Bouin's solution for 48 h. Livers were processed by routine methods (Luna 1960), embedded in paraffin, sectioned at 4 µm and stained with Gill's hematoxylin and eosin (H & E) for histologic examination.

Experiment 2. ***Protocol:*** Groups of 30 fry (3.2 ± 0.72 g) were exposed to static aqueous solutions of 0 (control) or 0.5 mg l^{-1} AFB_1 for 30 min. The fish were transferred to fresh water (without AFB_1) after carcinogen exposure. Subsamples of 5 fish from each group were each given [^{3}H]-TdR (specific activity = 55 Ci $mmol^{-1}$) i.p. as a dose of 5 µCi g^{-1} body wt (5 µl g^{-1} body wt, in sterile water) at 0, 1, 3, 7, or 14 d following carcinogen exposure. The fish were killed 24 h after [^{3}H]-TdR injection, livers were rapidly removed, cut in 2 slices with an alcohol-cleaned razor blade, and fixed in 1 % glutaraldehyde-1.5 % formaldehyde-0.01% picric acid in 0.1 M phosphate buffer pH 7.2, for 48 h at 4 °C. Fixed livers were dehydrated in graded alcohols, which were checked for residual radioactivity levels by liquid scintillation counting. Tissues were embedded in paraffin, sectioned at 4 µm, and placed on alcohol-cleaned glass slides.

Autoradiography: Sections were coated with Kodak NTB2 emulsion (diluted 1:1 with water) in a darkroom under safelight conditions (Kodak filter No. 2), placed in small, black boxes containing drierite, sealed with Scotch No. 33 photographic tape, and slides were kept for 6 wk at 4 °C.

Autoradiograms were developed under safelight

conditions in Kodak Dektol developer (1:1) for 2 min, rinsed in distilled water for 10 s, fixed in Kodak fixer for 5 min, and washed in distilled water for 5 min (Williams 1985). Slides were counterstained with H&E. In non-overlapping fields, a minimum of 100 hepatocytes were analyzed under high power (100×). Similarly, the percentage of 100 biliary cells showing label was determined. A nucleus with 5 or more grains was considered positive.

Chemicals. [^{3}H(G)] AFB_1 (Moravek Biochemicals, Inc., Brea, CA) was checked for purity by UV spectrometry and thin layer chromatography (TLC) (Loveland et al. 1983). [^{3}H]-TdR (55 Ci mmol^{-1}) was purchased from ICN Radiochemicals (Irvine CA). Phenol was purchased from Clontech Labs (Palo Alto, CA) and all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

RESULTS

Biochemical analysis

Results from in vivo [^{3}H]-AFB_1-DNA binding analysis are presented in Fig. 1. Linearity in [^{3}H]-AFB_1-DNA binding dose-response curve and a significant ($p < 0.001$) decrease in DNA binding in fish induced by BNF are shown. These results were found 24 h after carcinogen exposure at which time AFB_1-DNA binding has been reported to be maximum following i.p. administration (Goeger et al. 1986). It was also observed that dietary pretreatment with 500 mg l^{-1} BNF resulted in a decrease in AFB_1-DNA binding to a level approximately 20% of that in control fish.

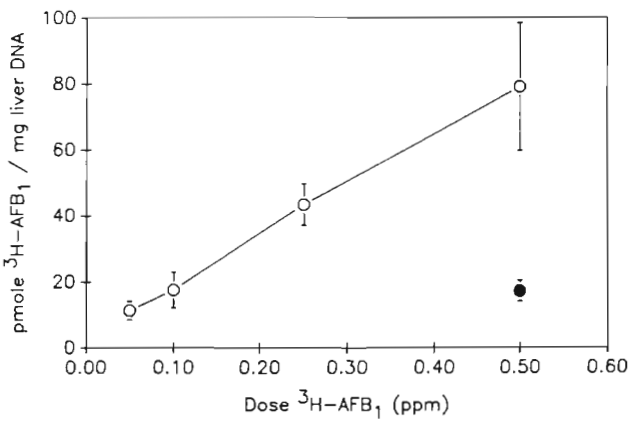

Fig. 1. *Oncorhynchus mykiss*. In vivo DNA-binding dose-response curve for aflatoxin B_1 (AFB_1) in rainbow trout fry. Data are means ± SD from 3 pools of 15 specimens each; correlation coefficient = 0.913. (●) β-naphthoflavone (BNF) pretreated trout, p-value < 0.001 from Student's t-test (Steel & Torrie 1980)

Histopathology

Hepatocellular alterations from rainbow trout 2 wk after exposure to [^{3}H]-AFB_1 are shown in Table 1. Livers, in which cytotoxic damage was seen microscopically, had a striking white color when examined macroscopically. Early cytotoxic changes, including nuclear and cytoplasmic swelling, were observable 1 wk after [^{3}H]-AFB_1 exposure, but by 2 wk, cytotoxicity was well advanced revealing increasing severity with increasing dose (Table 1).

Table 1. *Oncorhynchus mykiss*. Hepatocellular alterations in rainbow trout fry exposed 2 wk earlier to [^{3}H]-AFB_1

Dose (mg l^{-1})	Cytotoxicity Severe	Focal	Mild
0.5	8/8	0/8	0/8
0.25	4/11	4/11	3/11
0.1	0/10	0/10	0/10
0.05	0/8	0/8	0/8
0.5[a]	0/11	1/11	0/11

[a] Fry were fed 500 ppm BNF for 1 wk prior to exposure

Normal trout liver is organized in a tubular pattern (Hampton et al. 1985). Polygonal hepatocytes were organized as tubules 2 cells wide in longitudinal section but 5 to 8 cells formed the tubules in cross section (Fig. 2).

In the present study cytotoxicity was classified into 3 categories according to its severity (Table 1). Fish exposed to 0.5 mg l^{-1} [^{3}H]-AFB_1 presented severe cytotoxicity which was generalized throughout the liver The normal tubular architecture was no longer discernible due to hepatocyte swelling and necrosis. Surviving hepatocytes exhibited foamy cytoplasm and pleomorphic nuclei (Fig. 3). Small basophilic cells with scanty cytoplasm were seen interspersed among degenerating hepatocytes. In many cases, they appeared to originate from centrotubular regions, the location of bile ductule or presumptive oval cells. In contrast with rodent oval cells (bile ductule cells) of the canals of Hering, which are localized in periportal regions only, presumptive oval cells (bile ductule cells) in rainbow trout are localized throughout the liver in centrotubular (bile canalicular) locations (Hampton et al. 1988). Their size varied from very small with oval-shaped nuclei to larger polygonal cells with more rounded or irregularly shaped nuclei. The cells are presumed to be of similar origin so variability in size and shape may reflect age and degree of differentiation. Some of the cells occupied normal-appearing hepatic tubules, some formed ductules, while others

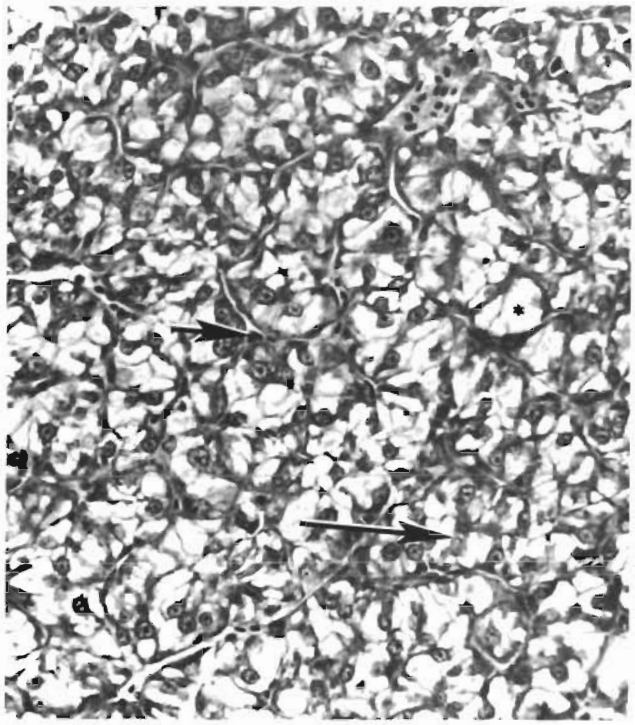

Fig. 2. *Oncorhynchus mykiss*. Normal trout liver. Hepatocytes are organized in a tubular architecture pattern, interspersed with sinusoids (short arrow). Tubules are 2 cells-thick in longitudinal section (long arrow). High glycogen (*) is a normal feature in young trout livers; H & E × 375

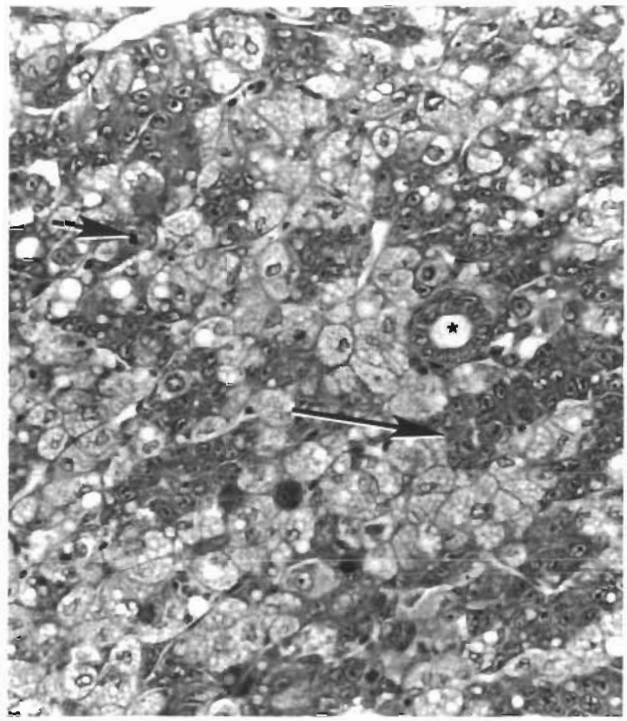

Fig. 3. *Oncorhynchus mykiss*. Severe cytotoxicity in a fish exposed 2 wk earlier to 0.5 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Liver architecture is disrupted, sinusoids are occluded due to dilation of hepatocytes which show foamy cytoplasm. Strongly basophilic cells, presumptive oval cells, (long arrow) are seen interspersed with degenerating or necrotic hepatocytes. Mitotic figures are frequent among basophilic cells (short arrow). Note the presence of an atypical ductule (★); H & E × 375

had not yet acquired any organizational substructure. Mitoses were observed frequently and cell proliferation was not localized to any particular region of the liver (Fig. 3). In spite of the severe liver damage no mortalities were found in fish exposed 2 wk earlier to 0.5 ppm [^{3}H]-AFB_1.

Focal cytotoxicity was seen mostly in livers from fish exposed to 0.25 mg l^{-1} [^{3}H]-AFB_1 (Table 1). Degenerative changes and necrosis in the focal areas were comparable with the severe cytotoxicity observed in the 0.5 ppm [^{3}H]-AFB_1 group. Swollen hepatocytes with foamy cytoplasm disrupted normal architecture. Other hepatocytes, though non-necrotic, showed toxic effects such as swelling, atypical nuclei, loss of glycogen, and accentuation of intercellular spaces (Fig. 4). Proliferation of presumptive oval cells was common in cytotoxic areas but not observed in non-necrotic parenchyma (Fig. 4).

Mild cytotoxicity was also seen in some livers from fish exposed to 0.25 mg l^{-1} [^{3}H]-AFB_1 (Table 1). Livers in this category retained tubular architecture but degenerating hepatocytes were found scattered throughout the liver. In addition, surviving hepatocytes revealed glycogen depletion and nuclear atypia. Proliferation of presumptive oval cells was not observed in livers presenting only mild cytotoxicity (Fig. 5).

Neither 0.1 nor 0.05 mg l^{-1} [^{3}H]-AFB_1 caused obvious cytotoxic responses in livers (Fig. 6) except that atypical nuclei (pleomorphism in both size and shape) were observed occasionally. These observations were not quantified and they are not mentioned in Table 1. Glycogen levels were comparable to controls.

Fish fed 500 ppm BNF for 1 wk prior to exposure to 0.5 ppm [^{3}H]-AFB_1 showed a significant ($p < 0.001$) decrease in cytotoxic response (Table 1). Normal liver architecture (Fig. 7) was found in all but one liver that showed focal cytotoxicity (Table 1).

Autoradiography

Labeled hepatocytes from control livers occurred infrequently and were predominantly located at the periphery of the liver (Fig. 8). Fish exposed to 0.5 ppm [^{3}H]-AFB_1 showed severe cytotoxicity at 2 wk, as was described in Expt 1. Mitotic indices in AFB_1-exposed

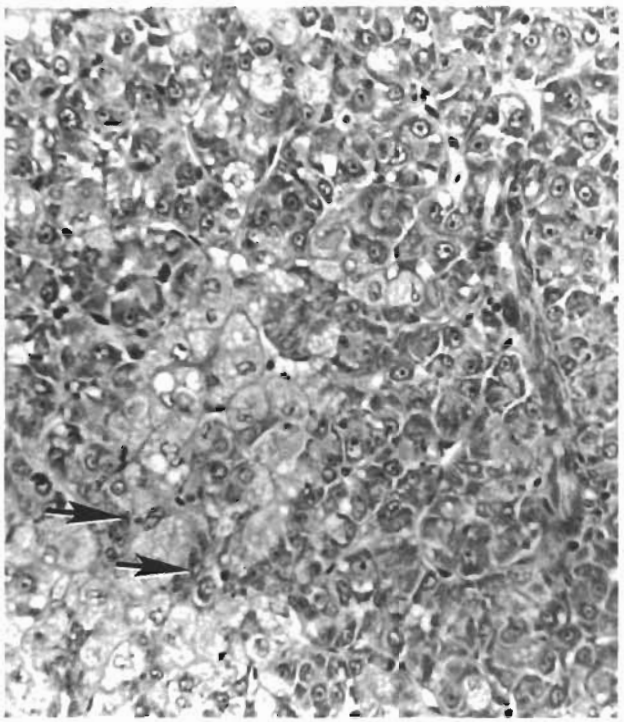

Fig. 4. *Oncorhynchus mykiss*. Focal cytotoxicity in a fish exposed 2 wk earlier to 0.25 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Degenerating hepatocytes with foamy cytoplasm are interspersed with presumptive oval cells (arrows). Non-necrotic hepatocytes (on the left) have greatly reduced glycogen content and some nuclear atypia; H&E × 375

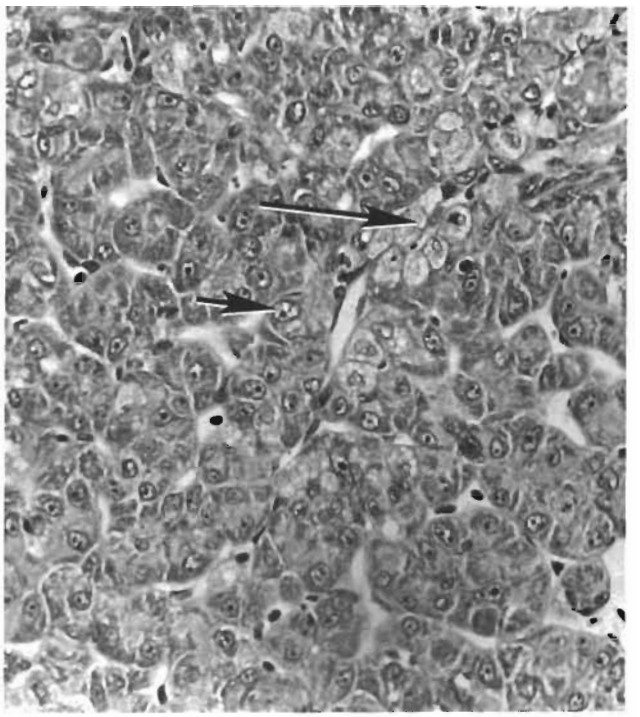

Fig. 5. *Oncorhynchus mykiss*. Mild cytotoxicity in a fish exposed to 0.25 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Swollen hepatocytes with foamy cytoplasm (long arrow) are seen scattered throughout the parenchyma. Nuclear atypia (short arrow) and reduced glycogen are also observed; H&E × 375

trout are shown in Table 2. No labeling of hepatocytes was observed, however, presumptive oval cells, which were proliferating intermixed with degenerating hepatocytes, were strongly labeled (Fig. 9).

DISCUSSION

DNA binding and hepatocarcinogenicity

Metabolism of AFB_1 to the electrophilic 8,9-epoxide, which binds covalently to macromolecules such as DNA, is required for AFB_1 carcinogenicity (Miller 1978). Consequently, DNA adduct formation can be used as an indicator of the carcinogenic potential of AFB_1 (Whitham et al. 1981, Dashwood et al. 1989). Results from the present study showed a linear dose-response in [^{3}H]-AFB_1-DNA binding following exposure of fry to static solutions of AFB_1 (Fig. 1). Even though none of these fish were maintained until tumors developed, the present AFB_1 treatments would be carcinogenic for the following reasons: (1) A recent experiment in our laboratory tested the carcinogenicity of a broader but similar range of AFB_1 doses (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 mg l^{-1}) in trout embryos and showed a dose-responsive carcinogenicity beginning at even the 0.01 mg l^{-1} level (J. Hendrick et al. unpubl. results). Other experiments have shown repeatedly a greater sensitivity of fry than embryos to a given applied concentration of carcinogen (J. Hendrick et al. unpubl. results); (2) The levels of AFB_1-DNA binding found in the present study are in the range of DNA adduction that results in a significant final tumor response (Dashwood et al. 1989). Thus, each of the doses used in the present short-term study would be expected to have a significant carcinogenic effect.

Cytotoxicity and hepatocarcinogenicity

The relationship between cytotoxicity and carcinogenicity is of considerable interest. Hoel et al. (1988) concluded, after comparing 2-yr rodent studies involving 99 chemicals, that a relatively small percentage of chemical carcinogens may act through a secondary mechanism such as cytotoxicity rather than a direct interaction with cellular DNA. In the present study, AFB_1 cytotoxicity also indicated a dose-dependent response (Table 1). A relationship between the metabolic activation of AFB_1 and cytotoxicity was

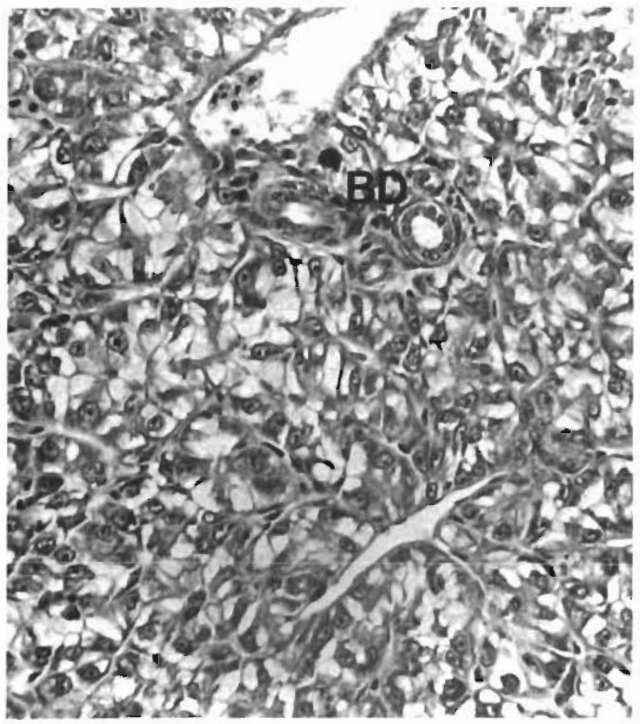

Fig. 6. *Oncorhynchus mykiss*. A normal liver in a fish exposed 2 wk earlier to 0.05 mg l^{-1} [^{3}H]-AFB_1 for 30 min. Hepatocytes are organized in a tubular pattern, with glycogen storage at normal levels. No signs of cytotoxicity are seen. A blood vessel and 2 bile ducts (BD) are present; H & E × 375

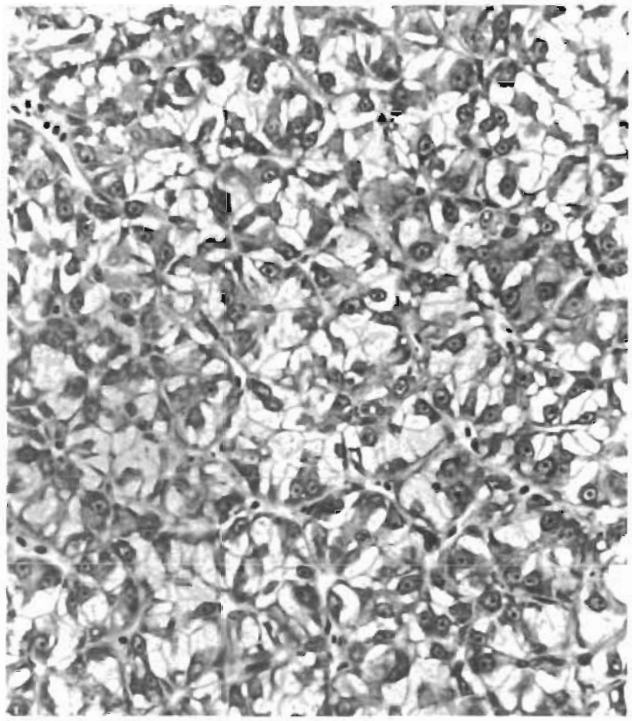

Fig. 7. *Oncorhynchus mykiss*. Trout liver in a fish fed 500 ppm BNF for 1 wk prior to exposure to 0.5 mg l^{-1} [^{3}H]-AFB_1 for 30 min and sacrificed 2 wk later. Lack of cytotoxicity is striking; H & E × 375

observed. Furthermore the results from BNF-induced fish, which showed a significant reduction in cytotoxic effects, and reduced DNA binding (which may indicate a reduced 8,9-epoxide formation) support the relationship between AFB_1 activation and cytotoxicity. Although AFB_1-DNA binding, which was used in this study as an indirect measure of the production of reactive AFB_1 metabolites, correlates well with cytotoxicity, it does not appear that DNA binding is linearly related to cytotoxicity. At least 2 sets of evidence support this conclusion. First, as stated earlier, each of the doses administered resulted in DNA binding, but only the 0.25 and 0.5 mg l^{-1} doses caused observable cytotoxicity. Thus, unlike DNA adduction or carcinogenicity, the cytotoxic response displays a threshold effect. It is possible, for example, that binding of the 8,9-epoxide to cellular macromolecules other than DNA at high doses may be necessary for cytotoxicity. Appleton et al. (1982) investigated AFB_1 binding in rat liver and determined the relative levels of binding in RNA, DNA, and protein. Consistently the levels of binding were greater in RNA, intermediate in DNA and lowest in protein. Second, cytotoxicity induced by other hepatotoxins such as bromobenzene (Reid & Krishna 1973) and acetaminophen (Jollow et al. 1973) seems to be linked

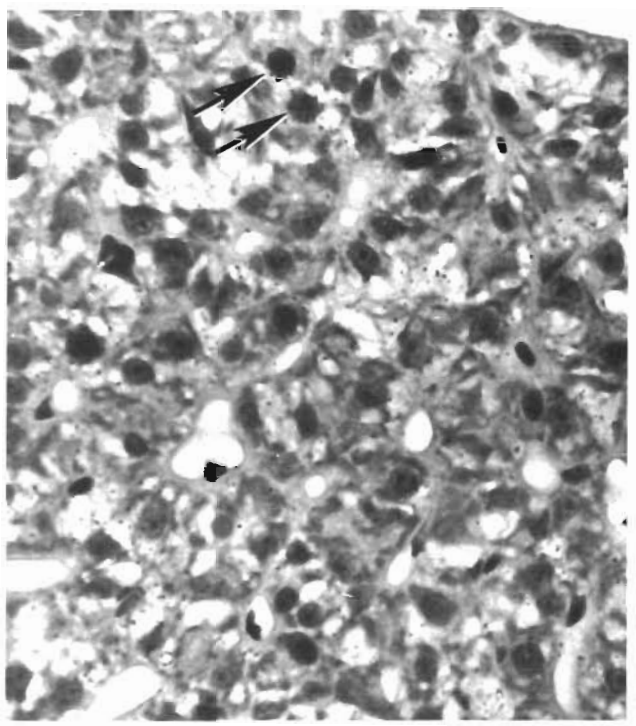

Fig. 8. *Oncorhynchus mykiss*. Control liver from a trout 24 h after i.p. injection of [^{3}H]-TdR. Strongly labeled hepatocytes (arrows) are seen at the periphery of the liver; H & E × 600

Table 2. *Oncorhynchus mykiss*. Mitotic indices[a] in AFB_1 exposed trout

Fish	Biliary epithelial cells[c]	Hepatocytes[d]
1	28.6 ± 5.5	–
2	9.3 ± 8.5	–
3	29.6 ± 6.6	–
4[b]	–	–

[a] Mitotic index = $\frac{\text{No. of labeled cells}}{\text{100 cells of that type}} \times 100$

[b] This fish showed severe necrosis which precluded identification of any cell type

[c] Results are mean of 3 countings ± SD

[d] No labeling was observed among degenerating hepatocytes

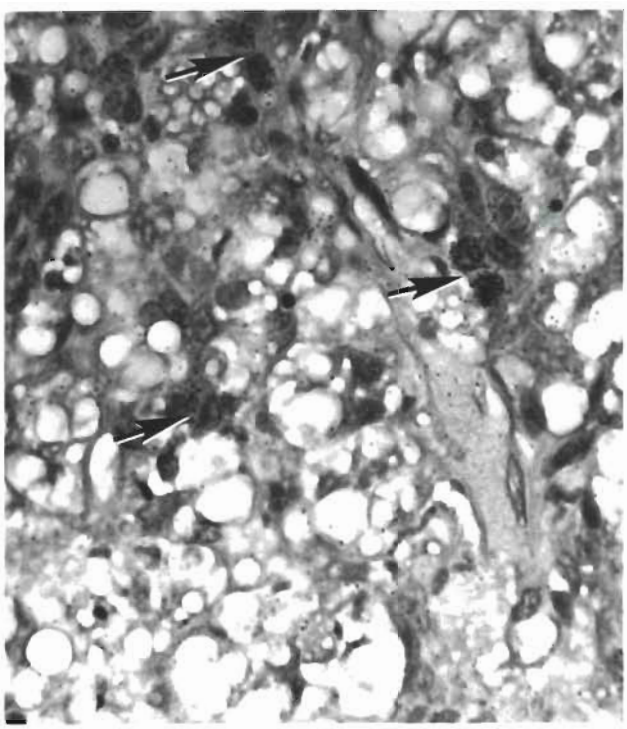

Fig. 9. *Oncorhynchus mykiss*. Liver from a trout exposed to 0.5 ppm AFB_1 for 30 min 2 wk before i.p. injection of [^{3}H]-TdR, and sacrificed 24 h later. Severe cytotoxicity is evident. Strongly labeled presumptive oval cells (arrows) are seen interspersed among degenerating hepatocytes; H&E × 500

primarily to cytoplasmic protein binding with only minimal nuclear binding. Thus at present we do not have definitive evidence on the critical macromolecular target(s) for AFB_1 binding or possible other mechanisms involved in cytotoxicity.

Target cells and hepatocarcinogenicity

Initiation of liver target cells is currently thought to require at least 2 steps (Columbano et al. 1981). These are covalent binding of an electrophile to DNA and subsequent fixation of the DNA lesion into the genome by cell replication. Cytotoxicity may be linked to increased carcinogenicity through compensatory cell proliferation (Figs. 2 and 3). Questions logically arise as to which cell type(s) in the liver is (are) initiated by chemical carcinogens. At least 3 possibilities exist: (1) primitive stem cells, (2) hepatocytes or (3) bile ductule (oval) cells. Lombardi (1982) concluded that there is no direct evidence for the existence of stem cells in the liver. It is generally accepted, at the present time, that hepatocytes are the critical target cells since they possess the highest activity of mixed function oxidase (MFO) enzymes and metabolize procarcinogens to electrophilic species (Farber et al. 1977). Initiation is thought to produce a few altered hepatocytes which become resistant to the cytotoxic and cytostatic actions of chemical carcinogens and can proliferate under the compensatory growth stimulus of necrosis or partial hepatectomy (Farber 1981). Such is the hypothesis explaining the rapid emergence of gamma-glutamyl transpeptidase (GGT) positive foci in rat liver after carcinogen exposure, partial hepatectomy and dietary exposure to the cytostatic chemical 2-acetylaminofluorene (2-AAF) (Solt et al. 1977). An alternative hypothesis is that the GGT(+) foci originate from oval cells, which inheritantly have traits similar to the proposed altered hepatocytes, i.e. GGT positivity and resistance to the cytotoxic/cytostatic properties of many carcinogens (Lombardi 1982). In an effort to determine which cells in the liver are initiated by carcinogens, Sell et al. (1981) fed rats a diet containing 2-AAF for 7 d along with multiple doses of [^{3}H]-TdR from Days 4 through 6. Rats were killed on the 7th day and livers, stained for GGT, were processed for autoradiography. There was no labeling of hepatocytes, but GGT(+) cells in the portal triad regions were heavily labeled and displayed frequent mitotic figures. These results show that oval cells were not only resistant to the cytotoxic action of carcinogens but appeared to be stimulated to replicate their DNA and proliferate. On the other hand, hepatocytes underwent regressive changes resulting in cell necrosis.

Although oval cells have a low mono-oxygenase activity (about 1 order of magnitude less than hepatocytes) (Lombardi 1982), and would be expected to result in a corresponding lower rate of DNA-adduction, their ability to survive and proliferate in the toxic environment of carcinogen exposure may make them more likely than hepatocytes to be the critical target cells of carcinogens. Since there is evidence that oval cells can differentiate into hepatocytes as well as bile ducts

(Grisham 1980), they could be the progenitors of both hepatocellular and cholangiocellular tumors of the liver (Hampton et al. 1988). Previous studies in our laboratory have shown that mixed carcinomas are the predominant tumor type in rainbow trout experimental carcinogenesis (Lee et al. 1989a, Nunez et al. 1989). In the present study, autoradiographs showed labeling of presumptive oval cells intermixed with degenerating hepatocytes which were not labeled (Fig. 9). These results are in agreement with those cited from rat studies and support the hypothesis that regeneration following cytotoxicity most likely originates with oval cells.

The hypothesis that oval cells are the target cells for carcinogen initiation and liver regeneration has greatest credibility when cytotoxic carcinogen doses are administered. Such doses are both cytotoxic and cytostatic to hepatocytes whereas oval cells may survive and proliferate either due to the compensatory demand created by hepatocyte loss or possibly by some unknown direct action of the carcinogen. The hypothesis has less credibility, however, when we consider non-cytotoxic doses of carcinogen. Lower doses of carcinogens would result in less DNA-binding to both hepatocytes and oval cells. Since there would be no hepatocyte loss, there would be no compensatory demand for proliferation. If 'initiated' hepatocytes are capable of mitosis, they would seem to be better candidates as target cells than oval cells under these conditions. The problem is that we know very little about the life span, differentiation and mitotic activity of normal, mature hepatocytes in fish. The source of normal liver growth or hepatocyte replacement is not well known. Whether new cells originate from mature hepatocytes, immature hepatocytes, bile ductule (oval) cells or stem-like cells has not been determined. We do not know if hepatocytes become post-mitotic and if so whether that condition is reversible or not. As a result, we do not know whether mature, differentiated, but 'initiated' (by DNA adduction) hepatocytes are capable of mitotic activity. Since trout liver is not arranged into lobules, as in mammals, it has been assumed that the trout liver is homogeneous throughout (Hampton et al. 1985, 1988). Based on iron staining and other histochemical stains, Lee et al. (1989b) suggested a peripheral zone of more immature hepatocytes in rainbow trout. Tritiated thymidine incorporation in normal livers in the current study was noticeably concentrated at the periphery of the livers as well. This may indicate that peripheral hepatocytes are more immature and mitotically active than more centrally located hepatocytes. If so this may relate to the observation that the vast majority of hepatocellular tumors in rainbow trout originate at or near the surface of the liver (Hendricks et al. 1984). We acknowledge that little is known about presumptive oval cells in fish (Couch & Courtney 1987, Hampton et al. 1988, Hinton et al. 1988). Morphologically, there appear to be cells that resemble mammalian oval cells, but the lack of positive zonation for identification and specific experiments to determine their properties causes their occurrence and role in carcinogenesis to remain speculative at the present time. Additional research in the areas of hepatocyte and oval cell biology in fish is needed to answer the questions generated by this work.

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