Enhancement of aflatoxin B₁ and N-methyl-N'-nitro-N-nitrosoguanidine hepatocarcinogenesis in rainbow trout *Salmo gairdneri* by 17-B-estradiol and other organic chemicals*

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ABSTRACT. Twenty-one-day-old rainbow trout *Salmo gairdneri* embryos were exposed to static solutions of aflatoxin B₁ (AFB₁) (0.5 ppm for 30 min) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (50 ppm for 60 min) to initiate neoplasms of the liver (AFB₁ and MNNG) and other organs (MNNG). Ten weeks after the onset of feeding (14 wk after carcinogen exposure) the fish were started on diets containing 500 ppm beta-naphthoflavone (BNF), 1000 ppm phenobarbital (PB), 100 ppm p,p'-dichlorodiphenyltrichloroethane (DDT), 1000 ppm indole-3-carbinol (I3C), or 20 ppm 17-beta-estradiol (E₂), (AFB₁-exposed fish) or PB, DDT and E₂ (MNNG-exposed fish) and fed until the experiment was terminated 12 mo after embryo exposure. E₂ was a significant enhancer of both AFB₁ and MNNG hepatocarcinogenesis. I3C, DDT and BNF also enhanced carcinogenesis to a lesser degree, and PB had no effect. Dietary BNF, PB and E₂ reduced the growth rate, while DDT, BNF and E₂ caused elevated mortalities. E₂ appears to have powerful enhancing or promoting properties on trout liver carcinogenesis, an observation that is consistent with elevated incidences of experimentally initiated hepatic neoplasms seen in sexually mature female salmonids.

INTRODUCTION

Earlier studies have demonstrated post-initiation enhancement of aflatoxin B₁ (AFB₁) hepatocarcinogenesis in rainbow trout *Salmo gairdneri* by dietary exposure to several organic compounds including cyclopropenoid fatty acids (CPFA), indole-3-carbinol (I3C) and beta-naphthoflavone (BNF). Feeding CPFA to rainbow trout, after embryo exposure to AFB₁, shortened the latency period and increased the final liver tumor incidence (Hendricks 1981). Dietary exposure of rainbow trout to BNF or I3C, following dietary AFB₁ initiation, led to a significant enhancement of final liver tumor incidence (Bailey et al. 1987a, Bailey et al. 1987b).

Peraino et al. (1975), Peraino et al. (1977) and Peraino et al. (1980) first demonstrated the enhancing effect of phenobarbital (PB) in 2-acetylaminofluorene (2-AAF)-

initiated rat hepatocarcinogenesis, when PB was administered after the carcinogen. The PB enhancing effect has also been demonstrated in rat using other initiators such as 2-methyl-N,N-dimethyl-4-aminoazobenzene (2-Me-DAB) (Kitagawa et al. 1979), diethylnitrosamine (DEN) (Diwan et al. 1985) and benzo(a)pyrene (BaP) after partial hepatectomy (Kitagawa et al. 1980). PB also enhanced both spontaneous and chemically initiated liver neoplasms in mice (Peraino et al. 1973, Ohmori et al. 1981). Unlike its promoting effects on carcinogenesis in rats and mice, PB was ineffective as a promoter of dimethylnitrosamine (DMN) hepatocarcinogenesis in Syrian golden hamsters (Stenback et al. 1986). p,p'-Dichlorodiphenyltrichloroethane (DDT) has also enhanced 2-AAF-initiated hepatic tumors in rats (Peraino et al. 1975).

The literature contains a number of reports on the possible enhancing effects of female steroid hormones on spontaneous and experimentally initiated liver tumors in fish and mammals as well as humans. Takashima & Hibiya (1972) and Takashima (1976) reported the occurrence of spontaneous hepatic and

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dermal neoplasms in sexually mature female salmonids. Experimentally, after oral administration of E_2 to adult *Salvelinus* hybrids, treated females showed a high incidence of dermal fibrosarcomas compared to treated males and controls (Takashima 1976). Sinnhuber et al. (1974) reported high mortalities related to massive liver tumors in sexually mature female rainbow trout previously fed aflatoxin M_1 (AFM₁) and suggested a sex hormonal stimulation of tumor development. Hendricks (unpubl.) observed a 3-fold higher incidence of hepatic tumors in sexually mature female rainbow trout (75%) exposed to dietary DDT than in comparably exposed males (25%). Yager & Yager (1980) demonstrated that oral contraceptive steroids (estrogens) can act as promoters of experimental hepatocarcinogenesis in rats after DEN initiation. In humans, several reports have described the occurrence of liver cell adenomas in women using contraceptive steroids (Edmondson et al. 1976, Christopherson & Mays 1977, Christopherson & Mays 1979, Desser-Wiest 1979, Nissen et al. 1979, Vana et al. 1979).

Based on this information, we were interested in determining the possible enhancing effects of E_2 and several other chemicals on previously initiated neoplasms in rainbow trout. AFB₁, a liver procarcinogen, and MNNG, a liver, stomach and kidney direct acting carcinogen, in rainbow trout, were chosen as initiating agents.

MATERIALS AND METHODS

Animals and diets. Eggs from Shasta strain rainbow trout *Salmo gairdneri* brood stock were fertilized and incubated in 12°C well water at the Food Toxicology and Nutrition Laboratory. Twenty-one days later, 14 groups of 200 eggs were selected for static embryo exposures to AFB₁ (6 groups in 0.5 ppm solutions for 30 min), MNNG (4 groups in 50 ppm solutions for 1 h) and water (4 groups sham-treated in water as controls). After treatment, each group of eggs was placed in an individual incubation compartment where hatching and yolk-sac absorption were completed 4 wk later. One hundred vigorous fry from each group were fed the Oregon test diet (OTD) (Hendricks 1982) for 10 wk post-swimup and then placed on diets containing the following organic chemicals. Single AFB₁-initiated groups were fed either control diet or control diet plus 500 ppm BNF, 1000 ppm PB, 100 ppm DDT, 1000 ppm I3C or 20 ppm E_2 for 38 wk until necropsy 12 mo after treatment. Single MNNG- or sham-treated groups were fed either the control diet or control diet plus 1000 ppm PB, 100 ppm DDT or 20 ppm E_2 as above.

Mortalities began to occur after 8 wk in the 3 groups receiving the 100 ppm DDT diet, and became unacceptably high (>20%) after 10 wk. These groups

were returned to the control diet for 6 wk until all mortalities stopped and vigorous feeding had resumed. At that time they were placed on a diet containing 25 ppm DDT, and received that level of DDT for the remainder of the experiment. Those groups receiving the E_2 containing diet also had an unacceptable mortality rate after 5 mo. These groups were fed the control diet for the remainder of the experiment.

The trout were housed in 3-foot (0.91 m) circular fiberglass tanks, receiving 13°C water at 3 gallons (11.4 l) min⁻¹, and were on a 12 h light, 12 h dark photoperiod. Monthly group weights were taken to monitor growth, and diet consumption was recorded to calculate diet efficiency.

At necropsy, fish were killed with an overdose of tricaine methane-sulfonate (MS-222) and weighed individually. Livers from treated and control groups were excised, weighed and examined under a dissecting microscope for surface tumors prior to fixation in Bouin's solution. Tissue samples of stomachs and abnormal kidneys were also taken from MNNG-treated groups and fixed in Bouin's solution. After fixation, livers were hand-sliced to verify external tumors and to locate internal tumors as previously described (Hendricks et al. 1984). Tumors, normal tissue and questionable areas were embedding in paraffin, processed by routine methods, sectioned at 5 µm and stained with H&E for histologic examination. Histological classification of liver tumors at our laboratory is never exhaustive due to the large number of animals used in our experiments and the large number of tumors per liver in most experiments. If a liver had no visible external tumor, a standard slice from the anterior region of the liver (just posterior to the hepatic veins) was taken from each fish to check the condition of normal tissue and discover any microscopic neoplastic lesions that may have existed. If 1 or 2 tumors were present, these were embedded for histology. If many existed per liver, only 2 or 3 were embedded to establish their histologic nature, and verify them as neoplasms.

Previous reports from our laboratory (Shelton et al. 1984, Bailey et al. 1987a, b) have lumped all hepatic neoplasms into a general hepatocarcinoma category for reporting incidences. Recently, more thorough investigation has revealed several types of neoplasms in the livers of trout exposed to various carcinogens. Two major types – those of hepatocellular and those of cholangiocellular origin – exist, but the hepatocellular forms constitute the vast majority of the neoplasms. Since it has been suggested by the National Toxicology Program that tumors of differing histomorphogenesis (i.e. hepatocytes vs biliary cells) should not be combined to arrive at incidence figures (McConnell et al. 1986), we have deleted the cholangiocellular neoplasms from the incidences recorded in Tables 1 and 2.

Furthermore we have arrived at the incidence values given in Tables 1 and 2 on the basis of the most advanced neoplasm present in the 2 or 3 observed per liver histologically. Large tumors were not always observed histologically. If not, we assumed they were hepatocellular carcinomas, and we may have erred in this assumption since some of them could have been mixed carcinomas. This would not have changed our results, however, since we lump these 2 categories together.

Liver tumors were classified according to the criteria established by Ward et al. in the 'Atlas on Neoplasms and Related Disorders in Fishes' (unpubl.). Tumor incidence data were compared statistically by means of Fisher's Exact Test (Brownlee 1965, Steel & Torrie 1980). Fish body weight data were compared by Fisher's protected LSD (Steel & Torrie 1980).

Chemicals. AFB₁ (Calbiochem, San Diego, CA) was checked for purity by UV spectrometry and thin layer chromatography (Loveland et al. 1983). MNNG, BNF, I3C, E₂ and PB were purchased from Sigma Chemical Company (St. Louis, MO). p,p'-DDT was purchased from Aldrich Chemical Company (Milwaukee, WI). All other chemicals used were reagent grade.

RESULTS

Of the 5 chemicals fed, 3 (BNF, PB and E₂) significantly depressed growth rates ($p < 0.001$) (Tables 1 and

2). The growth reduction due to E₂ was noteworthy since these fish received the hormone for only 5 mo and were on control diet for the remainder of the experiment. BNF, E₂ and DDT caused greater mortalities than observed in the controls. DDT caused the highest level of mortalities but did not reduce growth rate in the survivors. I3C caused neither reduced growth nor increased mortalities.

Tumor incidences in AFB₁ and MNNG embryo-initiated rainbow trout are shown in Tables 1 and 2, respectively. As in previous studies, AFB₁ produced only liver tumors (Hendricks et al. 1984) but MNNG embryo exposure resulted in liver, kidney and stomach tumors (Hendricks et al. 1980, Kimura et al. 1981, Bailey et al. 1984), at 12 mo. E₂ strongly enhanced both AFB₁- and MNNG-initiated liver tumor incidence ($p > 0.0001$). In addition, tumors observed at necropsy were more numerous and larger in the E₂-fed groups.

PB and DDT alone did not produce tumors, but a single hepatocellular carcinoma was observed in the E₂ controls. Previous studies have demonstrated the non-carcinogenicity of BNF and I3C to rainbow trout (Bailey et al. 1987a, b). I3C enhanced AFB₁ carcinogenesis ($p = 0.0146$) as has been previously reported (Bailey et al. 1987b) but the previously demonstrated enhancing effect of BNF (Goeger et al. in press) was not significant in this study. DDT enhanced the incidence of both AFB₁- and MNNG-initiated liver tumors, but was more

Table 1. *Salmo gairdneri*. Final body weight and hepatocellular tumor incidence at 12 mo after embryo initiation with 0.5 ppm AFB₁ and sham control

Dietary treatment	Final body weight ¹ (g)	Liver cell tumors of hepatocellular origin HCC &/or MC ² HCA ³		Incidence ⁴ (%)	p-value ⁵
AFB₁-initiated					
OTD ⁶	123 ± 45	56	0	56/96 (58)	—
OTD + 500 ppm BNF	84 ± 46**	47	2	49/82 (60)	0.4519
OTD + 1000 ppm PB	95 ± 31**	51	1	52/93 (57)	0.4916
OTD + 100 ppm DDT ⁷	145 ± 46*	45	0	45/62 (73)	0.0411
OTD + 20 ppm E ₂ ⁸	90 ± 32**	72	0	72/84 (86)	<0.0001
OTD + 1000 ppm I3C	119 ± 41ns	69	1	70/95 (74)	0.0146
Sham-control					
OTD ⁶	129 ± 30	0	0	0/96 (0)	—
OTD + 1000 ppm PB	95 ± 30**	0	0	0/95 (0)	—
OTD + 100 ppm DDT ⁷	130 ± 35ns	0	0	0/68 (0)	—
OTD + 20 ppm E ₂ ⁸	91 ± 34**	1	0	1/91 (1)	—

¹ Values are average ± SD

² Fish included in this category had either HCC and/or MC as the most advanced neoplasm

³ Fish included in this category had only HCA (BF) as the most advanced neoplasm

⁴ Incidence: No of total hepatocellular tumors/total No of fish examined

⁵ From Fisher's Exact Test when compared to control

⁶ OTD: Oregon test diet (Hendricks 1982)

^{7,8} These diets were modified as explained in the text

* $p < 0.05$

** $p < 0.001$

Table 2. *Salmo gairdneri*. Final body weight and incidences of tumors in individuals MNNG initiated as embryos

Dietary treatment	Final body weight (g) ¹	No of fish	Liver cell tumor of hepatocellular origin HCC &/or MC ²	HCA ³	Incidence (%) ⁴	p-value ⁵	NBL (%) ⁶	p-value ⁵	Stomach adenomas (%)	p-value ⁵
OTD ⁷	121 ± 38	96	31	3	34 (35)	—	13 (14)	—	33 (34)	—
OTD + 1000 ppm PB	90 ± 25 ^{••}	95	36	4	40 (42)	0.2118	4 (4)	0.0208	22 (23)	0.0601
OTD + 100 ppm DDT ⁸	127 ± 33 ^{ns}	67	39	0	39 (58)	0.0032	4 (6)	0.0954	26 (39)	0.3388
OTD + 20 ppm E ₂ ⁹	86 ± 33 ^{••}	81	72	1	73 (90)	<0.0001	6 (7)	0.1421	23 (31)	0.3694

¹ Values are mean ± SD
² Fish included in this category had either HCC and/or MC as the most advanced neoplasm
³ Fish included in this category had only HCA (BF) as the most advanced neoplasm
⁴ Incidence: no. of total hepatocellular tumors/total no. of fish examined
⁵ From Fisher's Exact Test when compared to control
⁶ NBL: nephroblastoma
⁷ OTD: Oregon test diet (Hendricks 1982)
^{8,9} Diets were modified as explained in the text
[•] $p < 0.05$
^{••} $p < 0.001$

effective in enhancing MNNG-initiated tumors. PB had no effect on the liver tumor response of either carcinogen but significantly reduced the incidence of nephroblastomas initiated by MNNG ($p = 0.0208$). No other significant effects on extrahepatic carcinogenesis were observed.

Neoplasms of strictly hepatocellular origin were hepatocellular adenomas (HCA) and hepatocellular carcinomas (HCC) (Figs. 1 and 2). HCC were the predominant neoplasms observed. Mixed carcinomas (MC), second in frequency to HCC, contained both hepatocellular and cholangiocellular elements (Fig. 3). Tumors in these 3 categories were combined as tumors of hepatocellular origin (Tables 1 and 2) according to the criteria outlined by McConnell et al. (1986). Liver tumor incidence was based upon tumors of hepatocellular origin only (Tables 1 and 2). A few livers had basophilic foci (BF) only, whereas BF were common in livers having other more advanced neoplasms (Fig. 4). We believe BF progress to HCA or eventually HCC, therefore, BF were included in the HCA category (Goeger et al. in press).

Neoplasms of biliary origin included cholangiomas and cholangiocarcinomas (Figs. 5 and 6). These neoplasms were infrequent in occurrence and were not included in the incidence values as explained by McConnell et al. (1986). Cholangiofibrosis, also a rare lesion, was characterized by atypical biliary epithelium and fibrosis (Fig. 7).

Lipid-rich foci were seen distinctly in all of the groups but the controls. The hepatocytes of these foci were enlarged due to the presence of lipid vacuoles (Fig. 8).

Pancreatic metaplasia was observed in both AFB₁- and MNNG-initiated groups. Pancreatic metaplasia of hepatocytes usually occurred in mixed tumors adjacent to cholangiolar components. The cells formed acinar units, contained zymogen granules, exhibited bizonal staining and had prominent nucleoli (Fig. 9).

Invasion of blood vessels by neoplastic hepatocytes was observed. The walls of the vessels being invaded showed marked hyperplasia of the muscle layers (Fig. 10).

Stomach adenomas and nephroblastomas, as well as hepatic tumors, were seen in MNNG-initiated fish. The occurrence of any one tumor type (liver, stomach or kidney) appeared to be independent, having no apparent effect on the occurrence of any other tumor type in that fish. Thus fish could have had all 3 tumor types, any combination of 2 tumor types or any single tumor type. Stomach adenomas were grossly observed as polyps or cauliflower-like protuberances on the mucosal surface, as described by Kimura et al. (1981). They were composed of an overgrowth of tall columnar, mucinous epithelial cells forming both surface

Fig. 1 *Salmo gairdneri*. Hepatocellular adenoma (right) in a rainbow trout exposed to 0.5 ppm AFB₁ as an embryo. Cells are basophilic, arranged in 2-cell-wide tubule/cords and are not invading or compressing the adjacent tissue. A basophilic focus is present on the left. H & E $\times 250$

Fig. 2. *Salmo gairdneri*. A well-differentiated trabecular hepatocellular carcinoma (left) from an individual exposed to 0.5 ppm AFB₁ as an embryo and fed a diet containing 1000 ppm PB. Note wide cords of basophilic cells, invasion of the adjacent tissue and mitotic figures. H & E $\times 730$

epithelium and sub-surface glands. The cells were well differentiated, mitotic figures were rare and no invasion of subepithelial tissues was observed, justifying a benign classification (Fig. 11).

Nephroblastomas were large, expanding laterally and ventrally to cause distortion of the fish's body. They exhibited incomplete and imperfect maturation into tubules, glomeruli, and fibrous stroma. The tumors

were not encapsulated and invaded normal surrounding tissue (Fig. 12).

DISCUSSION

Our results demonstrate that E₂ strongly enhances both AFB₁- and MNNG-initiated liver tumor incidence,

Fig. 3. *Salmo gairdneri*. Mixed hepatocellular carcinoma from an individual exposed to 0.5 ppm AFB₁ as an embryo and fed a diet containing 100 ppm DDT. Both hepatocellular and cholangiolar components exhibit neoplastic features. Note the mitotic figures. H&E $\times 250$

Fig. 4. *Salmo gairdneri*. Basophilic focus from an individual exposed to 0.5 ppm AFB₁ as an embryo and fed a diet containing 100 ppm DDT. Cells appear normal except for basophilia. H&E $\times 250$

when administered after carcinogen initiation. This documented action of the female hormone, E₂, also provides a possible explanation for the spontaneous or enhanced tumor incidences previously described in mature female salmonids (Takashima & Hibiya 1972, Sinnhuber et al. 1974, Takashima 1976).

At the present time, we do not know if this constitutes a true cancer promotion model system or not,

since the administered doses of both AFB₁, and MNNG, alone, caused significant incidences of hepatic tumors. We also do not know the mechanisms by which E₂ exerts its enhancing effects. Each group of fish receiving the E₂-diet had elevated mortalities and depressed growth compared to either the positive or negative controls. Previous unpublished results from our laboratory have revealed a positive correlation

Fig. 5. *Salmo gairdneri*. Cholangioma from an individual exposed to 0.5 ppm AFB₁ as an embryo and fed a diet containing 500 ppm BNF. Note the well defined nodule composed of ductular structures characterized by normal biliary architecture, lack of nuclear atypia and no invasion of adjacent tissue.
H&E $\times 100$

Fig. 6. *Salmo gairdneri*. Cholangiocarcinoma from an individual exposed to 0.5 ppm AFB₁ as an embryo and fed a diet containing 1000 ppm I3C. Cells show pleomorphic and hyperchromatic nuclei. Invasion of the adjacent tissue and frequent mitotic figures are seen.
H&E $\times 250$

between growth rate and hepatic tumor incidences. But in this case tumor incidences were enhanced despite growth depression.

Johnstone et al. (1978) demonstrated that exposures of newly hatched rainbow trout fry to a 20 ppm E₂-containing diet for 30 d caused phenotypic sex reversal resulting in an all female population. Our dietary treatment was delayed, however, and no effect on the male/

female sex ratio was noted in any of the E₂-exposed groups.

Post-initiation exposure to I3C enhanced AFB₁ carcinogenesis as was previously reported (Bailey et al. 1987b). These results confirm the enhancing effects of I3C on AFB₁ carcinogenesis. Mechanisms of this effect remain obscure.

DDT enhanced the incidence of both AFB₁- and

Fig. 7. *Salmo gairdneri*. Cholangiofibrosis from an individual exposed to 50 ppm MNNG and fed a diet containing 1000 ppm PB. Atypia of biliary epithelium and fibrosis are seen. H&E $\times 250$

Fig. 8. *Salmo gairdneri*. Lipid-rich foci from an individual exposed to 0.5 ppm AFB₁ as an embryo and fed a diet containing 100 ppm DDT. Note the enlarged hepatocytes due to the presence of lipid vacuoles. H&E $\times 100$

MNNG-initiated liver tumors but more significantly with MNNG than AFB₁. It required 6 wk for the trout to recover from the toxic effects of the 100 ppm DDT diet, evidenced by a resumption of vigorous feeding behavior and no further mortality. The fish tolerated the 25 ppm DDT diet well with no evidence of toxicity. It is noteworthy that the survivors were equal to or greater than the controls in body size, thus reduced

growth did not coincide with mortality for this compound or DDT selectively caused mortalities in the smaller individuals.

PB had no effect on the liver tumor response of either carcinogen but appeared to reduce the incidence of nephroblastomas initiated by MNNG. In contrast to rats and mice, neither rainbow trout (Elcombe & Lech 1978, 1979) nor marine fishes (Bend et al. 1973) are

Fig. 9. *Salmo gairdneri*. Pancreatic metaplasia of hepatocytes in a mixed carcinoma from an individual exposed to 50 ppm MNNG as an embryo and fed a diet containing 100 ppm DDT. Pancreatic cells form acinar units with zymogen granules and prominent nucleoli. H&E $\times 400$

Fig. 10. *Salmo gairdneri*. Invasion of a large hepatic vein by a trabecular hepatocellular carcinoma in an individual exposed to 50 ppm MNNG as an embryo. Note formation of potential tumor embolus. H&E $\times 250$

capable of cytochrome P-450 mediated microsomal enzyme induction by PB. This characteristic of fish could explain the failure of PB to enhance hepatic tumor response to AFB₁ and MNNG in this study. Similar results were observed in hamster where PB was ineffective as a hepatic enzyme inducer (gamma-glutamyltranspeptidase) as well as a promoter of DMN hepatocarcinogenesis (Stenback et al. 1986). The significance of the PB reduced incidence of nephroblas-

tomas initiated by MNNG is still unclear. Unlike its effect in rats (Shinozuka et al. 1982), PB also had a growth depressing effect on rainbow trout but mortalities were low.

In the present study, BNF did not enhance tumor incidence as greatly as observed in the past (Bailey et al. 1987a); fish were started on the diets 10 wk after swim-up. Thus there was a 14 wk delay between the exposure to AFB₁ or MNNG and the exposure to BNF

Fig. 11. *Salmo gairdneri*. A stomach adenoma from an individual exposed to 50 ppm MNNG as an embryo and fed 100 ppm DDT. Tall columnar mucinous epithelial cells forming both surface epithelium and sub-surface glands are seen. H&E $\times 63$

Fig. 12. *Salmo gairdneri*. Nephroblastoma from an individual exposed to 50 ppm MNNG as an embryo and fed 100 ppm DDT. Note incomplete and imperfect maturation of both tubules and glomeruli. H&E $\times 250$

or the other compounds. This prolonged delay may have reduced the effectiveness of BNF as an enhancer of carcinogenesis. Its toxicity at the 500 ppm level was revealed by reduced growth rate and elevated mortalities.

These studies indicate that E_2 , I3C and DDT have the potential to function as true promoters in the trout liver tumor model. Experiments using subcarcinogenic doses of AFB₁ or other carcinogens, and subsequent

exposure to appropriate doses of these compounds, will be needed to verify this hypothesis.

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