

Initiation, Promotion, and Inhibition of Carcinogenesis in Rainbow Trout

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The identification of etiological agents in feral fish neoplasia epizootics has been hampered in part by the lack of suitable fish models, and complicated by the likely existence of environmental agents which can act to stimulate or reduce population responses to genotoxin insult. The response of fish to tumor inhibitors and promoters, and the underlying mechanisms of modulation, have been studied in the rainbow trout model. Dietary treatment of trout with the compounds indole-3-carbinol (I3C), β -naphthoflavone (BNF), or the polychlorinated biphenyl (PCB) complex Aroclor 1254, before and during exposure to aflatoxin B₁ (AFB₁), was shown to reduce the final incidence of hepatocellular carcinoma after 12 months, compared to fish receiving AFB₁ only. By contrast, treatment of trout with BNF or I3C following AFB₁ initiation led to a significant enhancement of ultimate tumor response. Similarly, simultaneous treatment of trout with PCB and the carcinogen *N*-nitrosodiethylamine led to syncarcinogenic enhancement, rather than inhibition, of tumor response.

Mechanisms of inhibition of AFB₁ carcinogenesis by PCB, BNF, and I3C were investigated. PCB and BNF, but not I3C, are known to be strong inducers of trout cytochrome P448 and associated activities. Dietary induction by BNF or PCB was shown to be accompanied in isolated hepatocytes by considerably altered AFB₁ metabolism, and by significantly reduced rates of DNA adduct formation for all three agents. All agents differentially altered *in vivo* AFB₁ pharmacokinetics, enhanced bile elimination of AFB₁ as the aflatoxicol-M1 glucuronide, and significantly reduced peak levels of liver DNA adduct formation. No effects were seen on repair of AFB₁-DNA adducts, which was very slow in trout. Detailed studies demonstrated that glutathione detoxication of the AFB₁-2,3-oxide is not a significant pathway in trout fed control or inhibitor diets. The precise means by which I3C reduces adduct formation are presently unclear.

Introduction

Aquatic pollutants including polycyclic aromatic hydrocarbons and nitrogen heterocycles have been associated with elevated incidences of neoplasia in feral fish populations (1-3). However, direct demonstration of causal relationships remain to be established. While a number of studies have demonstrated the responsiveness of laboratory fish to carcinogens, most studies have concentrated on carcinogens such as aflatoxin B₁ (AFB₁) and *N*-nitrosodiethylamine (DEN), which have more relevance to neoplasia in man than in feral fish (4,5). An exception is the environmental pollutant benzo(a)pyrene (BP), which was recently shown to be carcinogenic to rainbow trout (6).

Identification of etiological agents in feral fish neoplasia is further complicated by the almost certain presence of compounds which may act as modulators (promoters, inhibitors, cocarcinogens) of environmental carcinogen response. Recent studies have begun to examine the possible effects on tumor response and mechanisms of action of carcinogenesis modulators in fish.

We review here the studies of carcinogenesis and its modulation in a convenient laboratory fish model, the rainbow trout. Most of these studies have examined modulation of AFB₁ hepatocarcinogenesis by polychlorinated biphenyls (PCBs) and other agents. AFB₁, a highly potent mycotoxin, was initially identified as a human carcinogen following its discovery as the etiological agent in outbreaks of hepatocellular carcinoma in trout hatcheries (7). Though it is a dietary rather than an aquatic pollutant, studies on AFB₁ carcinogenesis modulation in trout by PCB and other agents do serve to indicate the extent to which carcinogenesis by genotoxic pollutants may be modulated in fish, and the possible mechanisms through which modulation can occur. Clearly, there is a need for similar studies using BP and other carcinogens directly relevant to the aquatic environment.

Tumor Studies in Trout and Other Laboratory Fish Models

The response of rainbow trout and other laboratory fish models to known mammalian carcinogens has been recently reviewed (4,5,8,9), and will only be summa-

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rized here. Carcinogen classes tested have included: mycotoxins; polycyclic aromatic hydrocarbons (PAHs); nitrosamines, nitrosamides, and other alkylating agents; heterocyclic amines; steroids and cyclic lipids; chlorinated hydrocarbon pesticides; and azo compounds. Several species of small aquarium fish have been tested for carcinogen response. Early studies of the zebra danio (*Brachydanio rerio*) and the guppy (*Lebistes reticulatus*) (10-17), and later experiments with the Japanese medaka (*Oryzias latipes*), the species *Poeciliopsis*, and other aquarium fish (18-23), have revealed liver to be a major target organ and hepatocellular carcinoma to be the most prevalent tumor type in fish carcinogenic response. Hepatocellular carcinoma is also the most common neoplastic response in trout (9, 24-28), though other neoplasms, including nephroblastoma, gastric and swim bladder papillary adenoma, and fibrosarcoma can be induced, depending on carcinogen used, exposure route, and dose. For example, dietary exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induces only gastric papillary adenomas in rainbow trout, whereas embryonic or fry water exposure to a solution of MNNG also induces hepatocellular carcinoma, nephroblastoma, and swim bladder papillary adenoma (8). The malignancy of some of these neoplasms has been demonstrated by transplantation into isogenic hosts (29, and Hendricks and Bailey, unpublished results). A summary of all reported tissue lesions in aquatic animals resulting from controlled xenobiotic exposures has recently been compiled (30), and the histological progression of liver neoplasms in rainbow trout has been reviewed (31,32).

Modulation of Carcinogenesis in Rainbow Trout

The response of rainbow trout to modulators of carcinogenesis depends on the initiator (carcinogen) used, the nature and dose of the modulator, and the relative timing of initiator and modulator exposure. As shown in Table 1, experiment 1, dietary treatment with the synthetic BNF or the naturally occurring indole I3C before, during, and after AFB1 exposure reduces the tumor incidence of hepatocellular carcinoma at 12 months, compared to controls receiving AFB1 alone. This effect is also seen if I3C or BNF exposure is restricted to the period before and during AFB1 treatment. By contrast, exposure to these modulators after AFB1 initiation significantly enhances tumor response (experiment 4). Several additional compounds, including 17- β -estradiol, DDT, and cyclopropenoid fatty acids, can promote hepatocellular carcinoma and other tumor types, following initiation by AFB1 or other carcinogens in trout (25,36,37, and Hendricks and Bailey, unpublished results).

The ability of compounds to inhibit carcinogenesis in trout can be observed over a range of carcinogen doses. Experiment 2, Table 1, shows that coexposure of trout to AFB1 and the PCB Aroclor 1254 results in a parallel

shift in the AFB1 dose-response curve toward higher AFB1 dose. Higher doses of Aroclor have been shown to provide more extensive inhibition of AFB1 carcinogenesis (38). However, PCBs do not provide universal protection against all carcinogens in trout. As shown in experiment 3, coexposure to Aroclors 1254 or 1242 actually enhances DEN hepatocarcinogenesis in trout. In this context it is interesting that the incidence of neoplasias in English sole in the Puget Sound shows a negative correlation with PCB levels in the sediment (39), suggesting that the initiator(s) of these tumors may be susceptible to PCB anticarcinogenesis rather than enhancement. It is important to note that PCBs have not proven to be carcinogenic to trout, or to any other fish species to our knowledge (e.g., experiment 2 PCB control), nor have we yet demonstrated promotion of carcinogenesis in trout by these compounds.

Mechanisms of Tumor Modulation in Trout

The tumor results outlined in Table 1 indicate that modulation of the carcinogenic process can occur during the initiation or post-initiation (promotional) phases of tumorigenesis in fish, as in mammalian test systems. Mechanisms of promotion of carcinogenesis in trout are currently poorly understood, and are under active investigation in our laboratory. By comparison, mechanisms of anticarcinogenesis involving the initiation process have been more thoroughly studied in this model fish system. For example, several previous studies have demonstrated that PCBs and BNF (33,40-43), but not I3C (44), are potent inducers of cytochrome P448 and associated hepatic mixed function oxidase activities in trout. The following studies examined the extent to which these responses could be correlated with altered AFB1 pharmacokinetics *in vivo*, cellular activation and detoxication reactions, and formation and/or persistence of liver DNA adducts, as possible mechanisms for inhibition during the initiation phase of carcinogenesis.

The effects of dietary modulators on cellular carcinogen metabolism and mutagenesis can be readily assessed using freshly isolated trout hepatocytes in short-term culture (40,45-47). Hepatocytes have been isolated from trout preferred control diet or diet containing appropriate levels of BNF, PCB, or I3C, and incubated under standard conditions with [³H]AFB1. The results of these studies are summarized in Table 2. The known enzyme-inducing behavior of dietary BNF and PCB is accompanied by major changes in AFB1 metabolism. Both compounds cause a massive increase in the production of the relatively less carcinogenic metabolite aflatoxin M1 (AFM1), a substantial decrease in the production of the highly carcinogenic aflatoxicol (AFL), and a decrease of 40 to 50% in the rate of formation of DNA adducts, compared to control hepatocytes. The compounds were not equivalent, however, since only PCB elevated the hourly rate of total AFB1 metabolism. By comparison, dietary pretreatment with I3C caused little

Table 1. Modulation of hepatocarcinogenesis in rainbow trout by tumor promoters and inhibitors.

Experiment	Exposure protocol ^a	Tumor incidence (%) ^b	Reference
1. Inhibition, varying dose of inhibitor	AFB ₁ (dietary, 20 ppb, days 57-66)	45/118 (38)	(33)
	AFB ₁ , plus BNF (50 ppm, days 1-114)	21/117 (18)	
	AFB ₁ , plus BNF (500 ppm, days 1-114)	7/120 (6)	
	AFB ₁ , plus I3C (1000 ppm, days 1-114)	5/118 (4)	
	Control diet	0/118 (0)	
2. Inhibition, varying dose of carcinogen	AFB ₁ (1 ppb, 12 months)	27/121 (22)	(34)
	AFB ₁ (4 ppb, 12 months)	68/126 (54)	
	AFB ₁ (8 ppb, 12 months)	98/118 (83)	
	AFB ₁ (1 ppb) plus Aroclor 1254 (50 ppm)	14/120 (12)	
	AFB ₁ (4 ppb) plus Aroclor 1254 (50 ppm)	38/122 (31)	
	AFB ₁ (8 ppb) plus Aroclor 1254 (50 ppm)	88/118 (75)	
	Aroclor 1254 (50 ppm)	0/180 (0)	
	Control diet	0/120 (0)	
3. Cocarcinogenesis, different carcinogen	DEN (1100 ppm, 12 months)	12/118 (10)	(35)
	DEN (1100 ppm) plus Aroclor 1242	37/92 (40)	
	DEN (1100 ppm) plus Aroclor 1254	25/116 (22)	
4. Inhibition or promotion, with timing of exposure varied	AFB ₁ (20 ppb, weeks 9-12)	9/99 (9)	(5)
	AFB ₁ , plus BNF (500 ppm, weeks 1-12)	1/100 (1)	
	AFB ₁ , plus BNF (500 ppm, weeks 13-24)	30/100 (30)	
	AFB ₁ , plus I3C (2000 ppm, weeks 1-12)	1/99 (1)	
	AFB ₁ , plus I3C (2000 ppm, weeks 13-24)	51/100 (51)	

^aAFB₁ = Aflatoxin B₁, BNF = naphthoflavone, I3C = indole-3-carbinol, DEN = diethylnitrosamine.

^bAll tumor incidences were taken 12 months after initiation commenced.

Table 2. AFB₁-DNA binding and metabolite distribution in hepatocytes from trout fed control and inhibitor diets.

Diet ^a	Relative distribution of recovered unbound isotope ^b				DNA adducts ^c	Reference
	Polar	AFM ₁ + AFL-M ₁	AFL	AFB ₁		
Experiment 1						
Control	21 (12)	2.1 (.5)	18 (4.3)	45 (20)	1.52 (.19) ^c	(46)
BNF	29 (8.3)	31 (9)	6.2 (2.2)	33 (18)	0.96 (.4)	
Experiment 2						
Control	3.6 (.6)	1.6 (.3)	11 (1.2)	84 (1.5)	1.13 (.13)	(48)
PCB	5.8 (.5)	27 (3)	5.2 (.9)	62 (2.9)	0.66 (.19)	
Experiment 3						
Control	2.6 (.4)	1.8 (.2)	8.1 (.6)	86 (1)	0.86 (.06)	(49)
I3C	2.6 (.3)	3.8 (.9)	7.8 (.5)	85 (1)	0.67 (.03)	

^aDiets were 500 ppm BNF for 6 weeks, 100 ppm Aroclor 1254 for 12 weeks, or 2,000 ppm I3C for 7 weeks. n = 7, 5, and 9 for each dietary group in experiments 1, 2, and 3, respectively.

^bExpressed as a percent of total isotopes recovered as aflatoxins from HPLC.

^cExpressed as μmole AFB₁ adduct/g DNA/μg AFB₁ metabolized. All values in parentheses represent (± SEM).

perturbation in production of AFB₁ metabolites, but did cause a significant decrease in DNA adduct formation. Thus the three anticarcinogens appear capable of reducing initial AFB₁-DNA damage at the cellular level, but not by the same mechanisms.

At the whole organism level, alterations in cellular carcinogen metabolism, along with modified transport processes or other effects, may operate to alter carcinogen distribution and elimination kinetics. The effects of PCB, I3C, and BNF on AFB₁ pharmacokinetics in trout have been extensively studied (48,49, unpublished results). Figure 1 summarizes some of the results of these studies. The major effect of all three inhibitors is a significant increase *in vivo* in the rate of elimination of AFB₁ polar metabolites in bile, with I3C showing the smallest effect. (Effects of these dietary compounds on another potentially important pharmacokinetic pool, urine, were not included and are currently under study

in our laboratory.) As in the hepatocyte studies, effects of these compounds were not identical, but were studied at only one dose each, that showing inhibition in the tumor studies. Though pharmacokinetic studies are highly laborious, it would be interesting to know if differences reported here are consistently maintained over a range of dietary concentrations of the three anticarcinogens.

The structures of the AFB₁ metabolites found in rainbow trout bile have been previously established in our laboratory, and found to consist almost entirely of glucuronides of AFL and AFL-M₁ (50). Bile collected from the same fish depicted in Figure 1 was analyzed on HPLC as published (50). As summarized in Figure 2, alterations in the levels of bile radioactivity in fish fed control, BNF, PCB, and I3C diets were accounted for by increases in AFL-M₁ glucuronide, with I3C again showing the smallest effect.

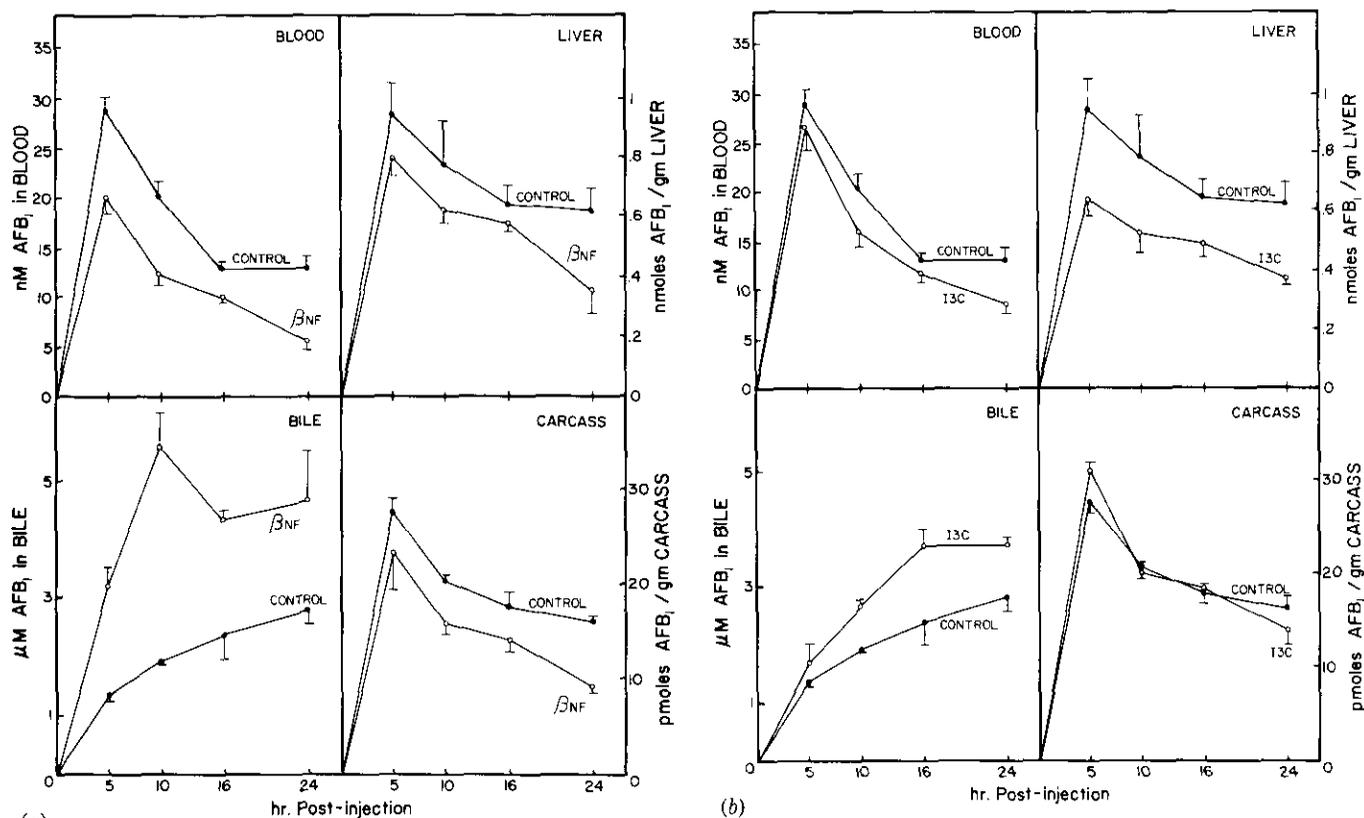
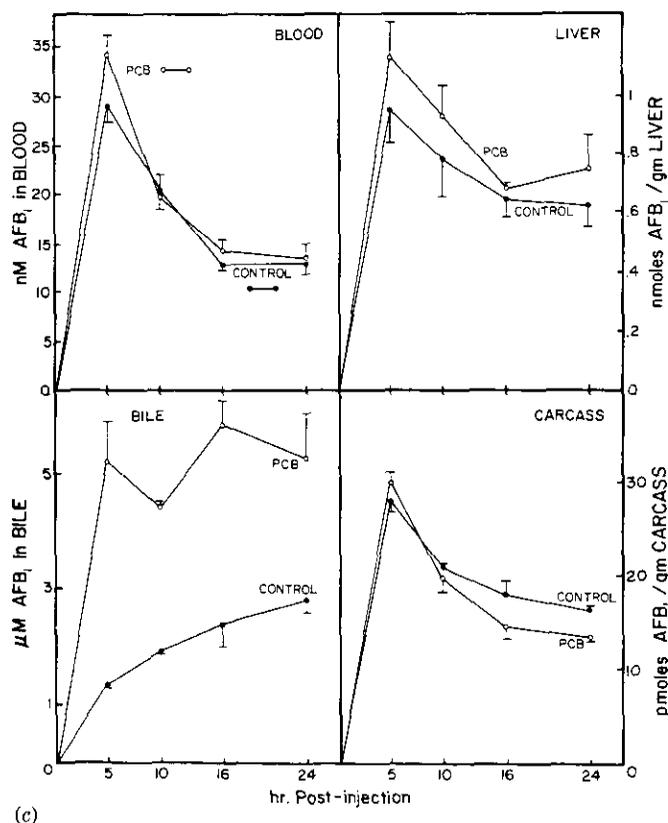


FIGURE 1. Effect of dietary pretreatment with (a) BNF, (b) I3C, or (c) PCB on AFB₁ pharmacokinetics in rainbow trout. Nine-month-old trout (55–75 g) were fed control diet or diet containing 100 ppm Aroclor 1254 (PCB), 2000 ppm I3C, or 500 ppm BNF for 8 weeks, then injected IP with [³H]AFB₁ (3.8 μCi/2.47 μg/25 μL ethanol). Three pools of three fish were sacrificed at 5, 10, 16, and 24 hr, and tissues collected and frozen in liquid nitrogen and stored at -60° C until analyzed. Tissue samples were solubilized in NCS (Amersham), acidified with acetic acid, and counted in OCS scintillant (Amersham) for tritium content. Each value is the mean of three groups of three pooled samples, except blood, where *n* = 9 individuals. Error bars represent SEM. These graphs are taken from previous publications (48) and (49) and are reproduced with permission.



We were concerned that our data did not indicate directly the extent of involvement of glutathione (GSH) conjugation as a detoxication pathway for AFB₁ in trout. This reaction may be especially significant since it traps the activated AFB₁-2,3-epoxide and prevents its interaction with DNA. Since only a small percentage of ingested AFB₁ (< 13%) forms liver DNA adducts, inhibitor-mediated alterations in even a minor amount of detoxication by this pathway could be significant. This reaction has further been implicated as significant in anticarcinogenesis and species differences to AFB₁ (51,52). To investigate more directly the possible role of this pathway in trout AFB₁ carcinogenesis and its modulation, authentic AFB₁-GSH conjugate was synthesized and used as an HPLC marker (53). Table 3 depicts the most significant results of these studies. Formation of AFB₁ was found not to exceed 1% of total AFB₁-derived metabolites in bile of trout fed control

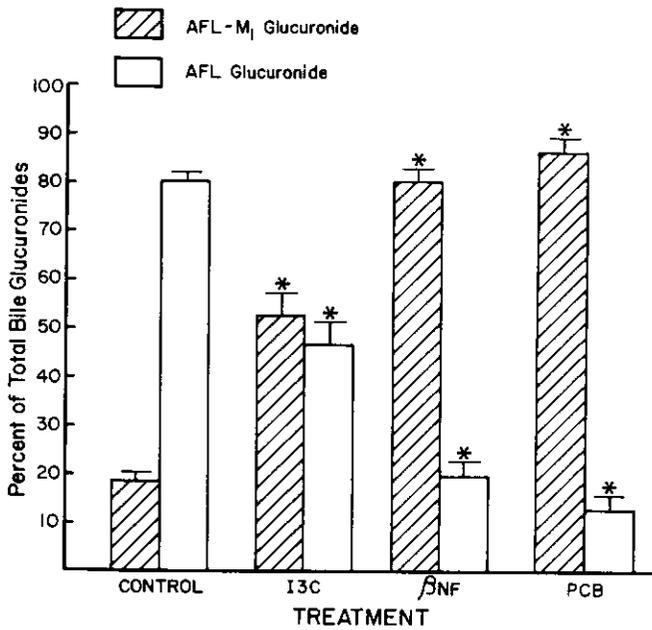


FIGURE 2. Bile aflatoxin metabolites from the gall bladder 24 hr after IP injection of AFB₁ into trout prefed PCB, BNF, or I3C. Samples were taken directly from fish treated in Fig. 1 and analyzed for AFB₁-derived metabolites as described (50).

Table 3. Effect of dietary modulators on aflatoxin B₁ glutathione and glucuronide conjugates in bile.

Diet ^a	Percentage of each metabolite in the HPLC profile ^{b,c}		
	AFB ₁ -SG ^c	AFL-M ₁ -g	AFL-g
Control	0.9 ± 0.8	16 ± 2.9	63 ± 3.1
BNF (500 ppm)	0.1 ± 0.1	88 ± 0.5	28 ± 2.8
I3C (2000 ppm)	0.1 ± 0.1	55 ± 7.7	36 ± 6.7
PCB (100 ppm)	ND	69 ± 6.0	18 ± 5.3

^aDiets were fed 3 weeks prior to AFB₁ injection.

^bAverage ± range (n = 2, with each sample being a pool of two individuals). Biles were taken 24 hr after IP injection of ³H-AFB₁.

^cAFB₁-SG = glutathione conjugate of AFB₁-2, 3-oxide; AFL-M₁-g = aflatoxicol-M₁ glucuronide; AFL-g = aflatoxicol glucuronide. ND = not detectable.

or inhibitor diets (Table 3), nor could it be produced in *in vitro* incubations using trout microsomes from any source, under conditions where mouse microsomes (or mouse-trout mixtures) produced large quantities of the conjugate (53). Further, we were able to demonstrate that diethyl maleate treatment could substantially deplete GSH levels in trout and coho salmon hepatocytes without significant effect on AFB₁-DNA adduct formation (data not shown). We conclude that GSH conjugation is not an important constitutive or inducible pathway for AFB₁ detoxication in rainbow trout. The involvement of this detoxication pathway for benzo(a)pyrene is under investigation in this species (Varanasi, personal communication).

The final questions to be investigated were whether the inhibitor-mediated reduction in DNA adduct formation observed in incubations with isolated hepato-

cytes would also be seen *in vivo*, and whether inhibitors might alter overall adduct persistence or repair. Liver nuclei were isolated from fish fed control, PCB, or I3C diets, and the DNA purified for determination of total level of adducts at various times after exposure. The results are depicted in Figure 3. As previously observed, peak adduct formation occurred in control trout 24 to 48 hr after AFB₁ exposure. Dietary pretreatment with each of the inhibitors significantly reduced the level of peak adduct formation compared to control. [Only the 24-hr data point has been studied for BNF, with a reduction of adducts to 44% of control (8)]. Surprisingly, I3C, the inhibitor with the weakest effects on enzyme

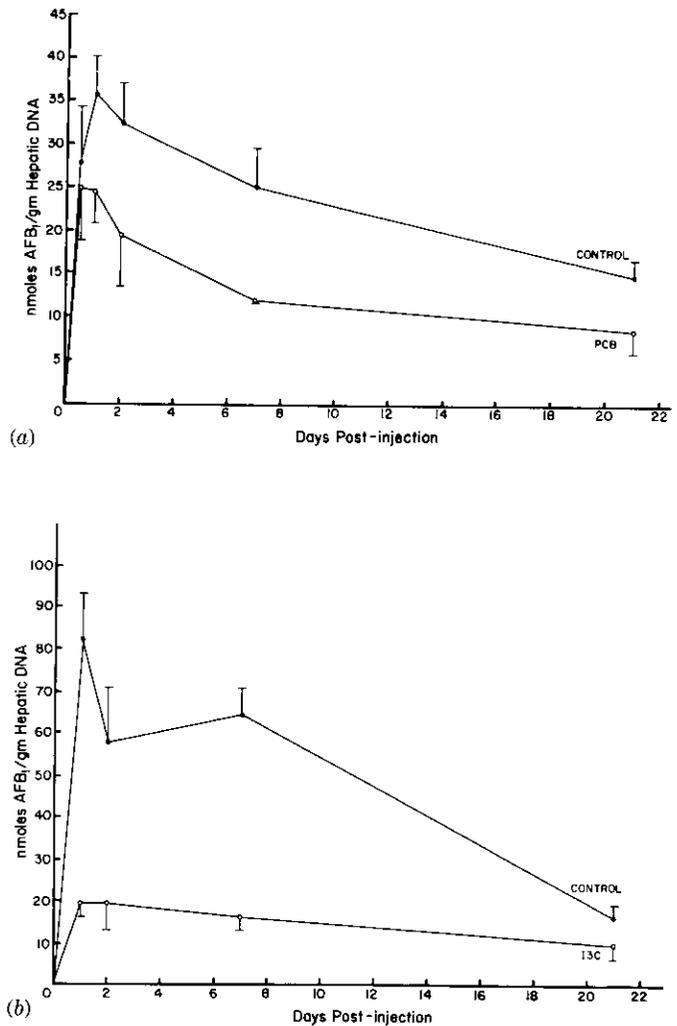


FIGURE 3. Effect of dietary pretreatment with (a) I3C or (b) PCB on the formation and persistence of AFB₁-DNA adducts *in vivo* in trout liver nuclei. Trout of 108 ± 23 g were prefed 100 ppm Aroclor 1254 or 2000 ppm I3C for 12 weeks, injected IP with [³H]AFB₁ (3.95 μCi/2.0 μg/100 μL ethanol), and sampled 1/2, 1, 2, 7, and 21 days later, without feeding. Liver nuclear DNA was purified and AFB₁-DNA binding determined as described (48). Data were weight-corrected to equal dosage. Each value represents the mean (±SEM) of three groups of three pooled individuals. These graphs are taken from previous publications (48) and (49) and are reproduced with permission.

induction, hepatocyte metabolism, or bile conjugate stimulation, had the strongest effect on reduction of DNA adduct formation *in vivo*. In no case was there any indication that these compounds alter the persistence of DNA adducts *in vivo*. Indeed, as previously reported (8), AFB1-DNA adducts were unusually persistent in trout liver compared to rates of rodent repair. This repair deficiency may account in part for the high sensitivity of this species to AFB1 carcinogenesis.

Summary

Chemical pollutants are thought to be responsible for tumor epizootics in a number of feral fish populations, but specific etiological agents have yet to be identified. Tumor studies in laboratory fish models provide one approach toward identification of carcinogens, and of environmental parameters which may influence the response of fish to genotoxins. Extensive evidence from tumor studies in mammalian models, and in the rainbow, suggest that a range of nongenotoxic dietary and environmental agents may intervene in the carcinogenesis process, to act as stimulators or inhibitors of tumor response. Although studies of tumor modulation with aquatic carcinogenic pollutants in fish models have not been conducted, extensive studies of modulation of AFB1 carcinogenesis in the rainbow trout may serve as a model for understanding mechanisms of modulation in fish. Studies in this system have shown that (a) a wide range of compounds can act as inhibitors, promoters, and co-carcinogens in trout; (b) particular modulators can act alternatively as inhibitors or promoters, depending on the carcinogen used and the relative timing of carcinogen and modulator exposure; (c) the magnitude of the effect does not appear to depend critically on carcinogens dose, but does depend on modulator dose.

Specific studies on the mechanisms of anti-initiation by BNF, I3C, and PCB for AFB1 carcinogenesis in trout have shown that dietary pretreatment by each of these three modulators leads to reduced initial formation of AFB1-DNA adducts *in vivo* and *in vitro*. The precise mechanisms by which this is achieved differ. BNF and PCB inhibit at least in part through induction of cytochrome P448, and associated enhancement of AFM1 and AFL-M1-glucuronide detoxication reactions. PCB also appears to enhance overall rates of AFB1 metabolism in intact trout and in isolated hepatocytes. I3C shows the weakest effects on these pathways but, at the doses studied, had the strongest effects on reducing DNA adduct formation. Addition of I3C itself does not alter AFB1 metabolism or DNA binding in control hepatocytes (49). Hence the mechanism(s) through which I3C-mediated AFB1 binding reduction occurs are not clearly understood, but may involve direct or indirect effects of I3C metabolites on AFB1 metabolism or transport.

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