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Enhancement of Hepatocarcinogenesis in Rainbow Trout with Carbon Tetrachloride

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Rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri) are sensitive to a number of chemical hepatocarcinogens, including mycotoxins, polynuclear aromatic hydrocarbons and nitroso-compounds (Metcalfe 1989). Liver neoplasms have been induced in trout using several different experimental protocols; including continuous dietary exposure of juvenile fish, and exposure of early life stages to chemical carcinogens.

Carcinogenesis studies with rodents have shown that liver tumor induction is a multi-step process (Farber and Sarma 1987). Liver neoplasms are rarely induced in adult rodents by a single exposure to chemical carcinogens. Induction usually requires repeated or continuous dosing (Godoy et al. 1976), or initiation by a single dose of a carcinogen followed by various promotional treatments (Solt et al. 1983; Peraino et al. 1980). A common element of many of the mammalian promotional models is restorative proliferation of the liver, which is usually induced by partial hepatectomy (PH) or by treatment with a hepatotoxic agent such as carbon tetrachloride (CCl_4). In humans, restorative liver proliferation induced by cirrhosis and hepatitis are major risk factors for liver cancer (Lieber et al. 1979; London 1980).

The purpose of this study was to determine whether, in the rainbow trout carcinogenesis model, proliferation of liver cells in response to post-initiation treatment with CCl_4 enhances liver tumor development. These data may be important in understanding the etiology of liver tumors in wild fish, where hepatotoxic chemicals with promotional properties may enhance tumor development (Malins et al. 1987), or hepatic pathogens may play a role in the development of tumors (Hayes et al. 1990).

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MATERIALS AND METHODS

Rainbow trout of the Kamloops strain were microinjected (0.5 μL volume) at the sac fry stage of development with DMSO (control) or 20 ng aflatoxin B_1 (AFB1) in DMSO, according to the methods described by Metcalfe et al. (1988). Following microinjection, half of the fish were not treated further and the remainder were repeatedly exposed to 1 mL/kg doses of CCl_4 by intraperitoneal (i.p.) injection. Thus, there were four treatment groups, referred to as DMSO, DMSO+CCl_4, AFB1 and AFB1+CCl_4.

Fish were dosed with CCl_4 at intervals of 21 d for a total of three doses before the 3-mo necropsy and seven doses before the 6-mo necropsy. The first i.p. injection occurred 25 d after initiation. The fish were weighed prior to each treatment, and were injected with a chromatography syringe at a position slightly anterior to the pelvic fin. Injection volumes ranged from 0.5 μL to 2 μL. Fish were anesthetized using CO_2 saturated water for the first two i.p. injections, and for the remaining injections, isoamyl alcohol was used as an anesthetic.

During the second i.p. injection with CCl_4, trout were marked by removing the adipose or anal fin, in order to identify the treatments. Fish from different treatments were reared together. Fish were fed ad libitum with a commercial trout diet (Martin Feed Mills, Ontario). The diet contained 40% crude protein, 12% crude fat, 3% crude fibre, 7,500 IU/kg of vitamin A, 3,000 IU/kg of vitamin D3, 100 IU/kg of vitamin E, and 800 mg/kg ascorbic acid. Fish were raised for up to 6 mo in filtered Otonabee River water (pH 7.8-8.2; alkalinity 60-80 mg $CaCO_3$/L; hardness 80-100 mg $CaCO_3$/L; temperature 9°-15°C).

At 3-mo and 6-mo post-initiation, trout were sacrificed by an overdose of anesthetic (MS-222) and necropsied. Fish were examined externally and internally for grossly visible neoplasms, and the liver was examined and preserved in Bouin's fixative for histological examination. The livers were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E). Livers in which lesions had been observed grossly (visual survey) were extensively sectioned for histological confirmation of visual observations. All other livers were surveyed by cutting 5-6 sections from the center of each paraffin block and examining the sections microscopically for evidence of neoplasia (histological survey).

The frequencies of hepatic neoplasms in trout were compared statistically by means of Chi-square contingency analysis with a continuity correction (Armitage, 1971). The data from the visual and histological surveys of neoplasm incidence were analyzed separately, since these survey methods were independent of each other. The fit of tumor multiplicity data to Poisson and negative binomial distributions was determined as described by Drinkwater and Klotz (1981).

RESULTS AND DISCUSSION

Preliminary tests with different i.p. doses of CCl_4 indicated that a 1 mL/kg dose induced approximately 16% mortalities per treatment. Examination of sections of liver tissue from trout sacrificed 2 wk after injection with CCl_4 revealed that there was focal necrosis of hepatocytes and numbers of mitotic figures were elevated throughout the liver.

No pathological lesions were seen on any of the internal organs except the liver. Liver neoplasms were observed grossly at both the 3-mo and 6-mo necropsies in treatments with AFB1 initiation. These lesions were all later confirmed histologically as hepatocellular carcinomas. The liver lesions observed grossly at the 3-mo necropsy were usually relatively small (<1 mm), while lesions observed grossly at 6-mo were larger (1-10 mm). No grossly-visible liver lesions were observed in the DMSO and DMSO+CCl_4 groups at 3-mo or 6-mo. Only single tumors were generally observed grossly in the AFB1 group, whereas in the AFB1+CCl_4 group, both single and multiple (2-4) tumors were observed grossly on the livers.

The histological classification of liver lesions was based upon the criteria outlined by Hendricks et al. (1981). Eosinophilic cell foci and vacuolated cell foci were identified in liver tissue, but they were not included as preneoplastic lesions. Basophilic cell foci and basophilic adenomas were commonly observed, and were both classified as preneoplastic lesions. The basophilic adenomas were distinguished from foci on the basis of their larger size (>0.5mm). Two cholangiomas were observed in one liver section from the AFB1+CCl_4 group at 3-mo. These lesions are considered benign bile-duct tumors (Hendricks et al. 1981), and were not enumerated as neoplasms. Basophilic hepatocellular carcinomas from 1-10 mm in diameter were noted histologically at both necropsies. In the smaller carcinomas, hepatic sinusoids

were distorted in a trabecular pattern, whereas larger carcinomas had a trabecular periphery and a central fibrous stroma. One lesion classified as a poorly differentiated liver hepatocarcinoma (Hendricks et al. 1981) was identified in the AFB1+CCl_4 group at the 6-mo necropsy, and was characterized by slight eosinophilia, large pleomorphic nuclei, and abundant mitotic figures. Carcinomas of this type are believed to be an advanced stage in the carcinogenic progression, as compared to the trabecular hepatocarcinoma.

Table 1. Numbers and incidence (%) of preneoplastic and neoplastic hepatic lesions observed at the 3-mo and 6-mo necropsies in rainbow trout microinjected with DMSO (Control) or AFB1 (20 ng/sac-fry), and treated with repeated i.p. injections of CCl_4 (1 mL/kg body weight).

	VISUAL SURVEY	HISTOLOGICAL SURVEY		
	Fish with Gross Neoplasms	Fish with Basophilic Cell Foci	Fish with Basophilic Adenomas	Fish with Basophilic Carcinomas
a) 3-mo necropsy				
DMSO (n=100)	0	0	0	0
DMSO+CCl_4 (n=100)	0	0	1(1%)	0
AFB1 (n=150)	4(2.7%)	14(9.3%)	7(4.7%)	19(12.7%)
AFB1+CCl_4 (n=165)	7(4.2%)*	9(5.5%)*	3(1.8%)*	40(24.2%)*
b) 6-mo necropsy				
DMSO (n=103)	0	0	0	0
DMSO+CCl_4 (n=82)	0	0	0	0
AFB1 (n=158)	16(10.1%)	15(9.5%)	5(3.2%)	38(24.1%)
AFB1+CCl_4 (n=131)	32(24.4%)*	11(8.4%)	3(2.3%)	35(26.7%)

* Incidence significantly different from AFB1 group ($p<0.1$).

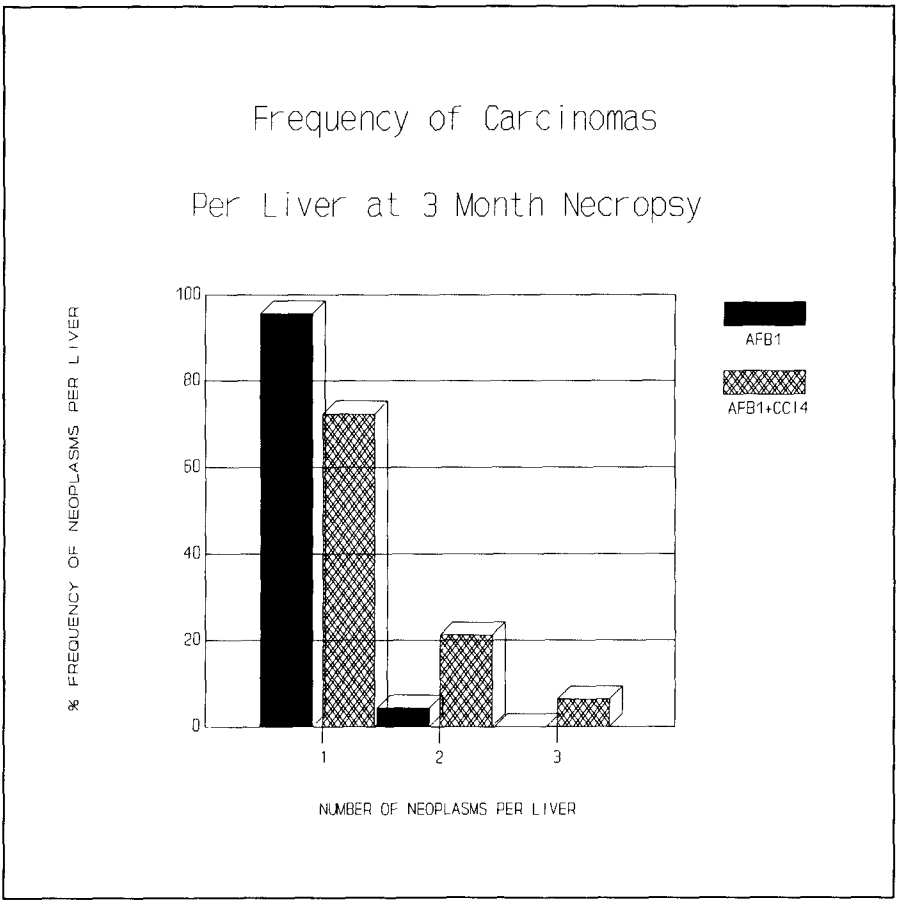

Figure 1. The frequency with which various numbers of tumors were observed per liver section in the AFB1 and AFB1+CCl_4 groups at 3-mo post-initiation.

At the 3-mo necropsy, the incidence of gross neoplasms observed in the visual survey and the frequency of hepatocellular carcinomas observed in the histological survey were significantly greater in the treatment with AFB1+CCl_4 than in the treatment with AFB1 initiation alone (Table 1). In contrast, the incidence of preneoplastic basophilic lesions observed in the histological survey was significantly lower in the AFB1+CCl_4 treatment. There was no significant difference between AFB1 and AFB1+CCl_4 treatments in the total incidence of basophilic lesions (preneoplastic plus neoplastic) observed in the histologic survey. At 6-mo, the AFB1+CCl_4 group showed a significantly higher incidence of grossly visible hepatic neoplasms in comparison to the AFB1 group (Table 1). However, in the histological survey, the incidence of preneoplastic and neoplastic lesions were not significantly different from the AFB1 group.

Figure 1 shows the frequency with which various numbers of carcinomas were observed at 3-mo in each liver section from the AFB1 and the AFB1+CCl_4 groups. Two and three carcinomas per liver section were observed more frequently in fish treated with AFB1+CCl_4. A Chi-square test indicated that multiplicity data for the AFB1 group followed a Poisson distribution, and therefore the presence of one tumor in the liver section did not enhance the probability of observing other tumors. In contrast, the multiplicity data for the AFB1+CCl_4 group followed a negative binomial distribution, and the probability of observing multiple tumors in the liver section was enhanced by the presence of the first. Multiple tumors per liver were also observed frequently in the histological survey at the 6-mo necropsy. The data indicate a slightly greater frequency of multiple tumors in the AFB1+CCl_4 group than in the AFB1 group, but this difference was not statistically significant.

In this study, we demonstrated that multiple treatments with CCl_4 enhanced AFB1-initiated hepatocarcinogenesis in trout over a short latency period (3 mo). An obvious enhancing effect by multiple treatments with CCl_4 over a longer latency period (6 mo) was not seen. In these experiments, the effect of CCl_4 on AFB1-initiated tumor development cannot be described as tumor "promotion". True promotion is the elevation of a subcarcinogenic response to a carcinogenic response by post-initiation treatments. Since exposure to AFB1 alone induced tumors in this experiment, the elevated response after CCl_4 treatment is described as tumor "enhancement".

Proliferation of hepatic cells is believed to be an essential part of liver carcinogenesis. For instance, a single injection of dimethylnitrosamine (DMNA) to adult rats did not induce hepatic neoplasms, but hepatic carcinomas did develop when rats received PH before exposure to DMNA (Craddock, 1976). When DMNA was administered as a single injection to rapidly growing newborn rats, liver tumors developed (Peraino et al., 1984). Farber and coworkers (Solt et al., 1983; Farber and Sarma, 1987) have shown that two episodes of cell proliferation are required for hepatic carcinogenesis in promotional models with adult rats. The first episode, which is often induced by PH before exposure to an initiating agent, allows DNA damage to become "fixed", and the second episode of proliferation, which is often induced by PH, or CCl_4 (hepatotoxic agent), or α hexachlorocyclohexane (mitogenic agent) promotes the progression of initiated cells to malignancy. In this study with trout, a first round of hepatic cell proliferation occurred naturally because of the rapid growth of the fish fry. The second round of cell proliferation was induced by CCl_4.

In the rainbow trout carcinogenesis model, dietary exposure of trout to DDT, β-napthoflavone, and indole-3-carbinol has been shown to enhance tumor development after AFB1 initiation (Bailey and Hendricks, 1988), but the mechanisms behind this activity have not been fully elucidated. Nuñez et al (1989) observed both promotion and enhancement of AFB1-initiated carcinogenesis when trout were exposed to two dietary concentrations of 17 β-estradiol. In this case, the mechanism for tumor enhancement was identified as mitogenic stimulation of cellular proliferation in the liver. CCl_4 appears to enhance tumor development in the rainbow trout model by a similar mechanism, although cellular proliferation occurs as a restorative response to the necrogenic activity of this chemical.

These data indicate that restorative cell proliferation is an important factor in the promotion of liver carcinogenesis in fish. Thus, exposure of wild fish to weak carcinogens in association with hepatotoxic chemicals or pathogens may lead to liver tumor development. These data also show that liver carcinogenesis in fish and mammals are modulated by similar factors.

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