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The project was designed to expand the usefulness and exploit the advantages of small fish carcinogenesis models. Studies have focused on two primary species, the Japanese medaka (Oryzias latipes) and the guppy (Poecilia reticulata). This report, arranged in referenced publication format, describes the following studies: (1) carcinogenesis tests with the halogenated hydrocarbon 1,1,2,2-tetrachloroethane (TeCE), (2) the heavy metal cadmium, and (3) the aromatic amine 2-acetylaminofluorene (AAF); hepatic metabolism studies with (4) AAF and (5) ethylene dibromide (EDB); and the occurrence of (6) thymic lymphoma and (7) acinar cell carcinomas of the exocrine pancreas.

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The project has already made several important contributions to the understanding of small fish carcinogenesis models. Carcinogenesis tests with TeCE and cadmium were negative. Possibly, the small fish are incapable of metabolizing some halogenated hydrocarbons to their carcinogenic intermediates. The cadmium test will be repeated using injection rather than water-borne exposure. Studies with AAF yielded interesting results. AAF was not carcinogenic to medaka but was to the guppy. Metabolic studies performed on the medaka suggest that it is more efficient in detoxifying AAF than it is in producing the carcinogenic metabolites. We now plan to examine the metabolism of AAF in the guppy and compare it with the medaka. Studies on the hepatic metabolism of EDB in medaka showed that it induces the phase II enzyme glutathione S-transferase which is involved in production of the ultimate carcinogenic species of EDB. Studies on thymic lymphoma and acinar cell carcinomas of the exocrine pancreas in medaka focused on understanding the pathology and progression of these lesions and, as relatively rarely occurring tumors, how they would affect the interpretation of large scale carcinogenesis bioassays with small fish.

FOREWORD

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William E. Hamilton Dec 14, 1989
PI Signature Date

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DEVELOPMENT OF CARCINOGENESIS BIOASSAY MODELS: RESPONSE OF SMALL FISH SPECIES TO VARIOUS CLASSES OF CARCINOGENS

Mid-term Report
December 14, 1989

I. Introduction

The use of fish models for carcinogenesis bioassays has gotten considerable attention in recent years (Dawe et al., 1981; Hendricks, 1982; Black, 1984; Hoover, 1984; Couch and Harshbarger, 1985; Mix, 1986; Hawkins et al., 1988; Powers, 1989). Small fish carcinogenesis models are being developed with two related applications in mind. First, small fish could be used and, in some limited cases have been used, to examine the carcinogenicity of water-borne and sediment-bound compounds that have been implicated in the occurrence of cancer of the liver and other organs in wild fishes as has been reported in several locations including winter flounder (Pseudopleuronectes americanus) from the Boston Harbor (Murchelano and Wolke, 1985) in English sole (Pleuronectes vetulus) from the Puget Sound (Malins et al., 1985), and in brown bullhead catfish (Ictalurus nebulosus) in several inland waterways (see Baumann, 1989). For example, the polynuclear aromatic hydrocarbon benzo(a)pyrene (B(a)P) is known to be carcinogenic in mammals where it mainly causes skin tumors when applied topically. The ubiquitous presence of B(a)P in sediments associated with cancer in those fishes brought to the forefront the question of whether it and associated compounds caused the hepatic and other lesions. Tests with waterborne exposures proved that B(a)P was hepatocarcinogenic to two species of small fishes, the Japanese medaka (Oryzias latipes) and the king cobra guppy (Poecilia reticulata) (Hawkins et al., 1989). Indeed, both B(a)P and a related polynuclear aromatic hydrocarbon, 7, 12-dimethylbenzanthracene appear to be far more carcinogenic in small fish models than in mammalian models (Hawkins et al., In Press).

The second principal application of small fish models concerns their biomedical potential for supplementing or, in some cases, replacing rodent models in carcinogenesis bioassays. Simon and Lapis (1984) illustrated this potential when they used bioassays with guppies to identify carcinogenic isomers of a series of chemotherapeutics that had nitrosamine moieties. In unpublished studies at our laboratory, several halogenated hydrocarbons were tested individually and in mixtures with medaka, guppies, and sheepshead minnow (Cyprinodon variegatus). In some respects, those test protocols which involved chronic exposures at stable concentrations (Walker et al., 1986) were superior to rodent bioassays in which volatile compounds such as halogenated hydrocarbons are given by gavage or in the drinking water.

This project was designed to facilitate the development of small fish carcinogenesis bioassay models, specifically the guppy and medaka, by identifying the classes of carcinogens that the models could detect. At the midway point of the contract, we have conducted bioassays of the halogenated hydrocarbon 1,1,2,2-tetrachloroethane (TeCE) against the medaka and guppy, the heavy metal cadmium against the medaka, and the aromatic amine 2-acetylaminofluorene (2-AAF) against the medaka and guppy. Studies on the metabolism related to carcinogenesis have been conducted 2-AAF and ethylene dibromide in the medaka. An ancillary aim of this project was to examine factors such as the occurrence of rare tumors that affect the interpretation of the results of small fish carcinogenesis bioassays. In the medaka, these tumors include thymic lymphomas and acinar cell carcinomas of the exocrine pancreas.

This report is presented as a series of individual studies in format similar to that used for articles submitted for publication in the referenced literature as suggested in Section F.4b of the contract reporting instructions.

II. Carcinogenesis bioassay with the halogenated hydrocarbon 1,1,2,2-tetrachloroethane on the Japanese medaka (Oryzias latipes) and the king cobra guppy (Poecilia reticulata)

A. Introduction

1,1,2,2-Tetrachloroethane (TeCE) is a solvent used in cleaning processes and in the manufacture of paints, varnishes and rust removers. TeCE is carcinogenic when administered by gavage to B6C3F1 mice in which is causes hepatic adenomas and carcinomas in the female and adenomas only in the male, but is not carcinogenic to F344 rats (Haseman et al., 1984). Because TcCE is a contaminant of ground supplies of drinking water, it is considered a potential health threat and is on EPA's Priority List of Drinking Water Contaminants (EPA, 1988).

We conducted carcinogenesis bioassays with TeCE against the medaka and the guppy in flow-through exposures. Histopathological examination of specimens exposed to TeCE for three months then grown-out in clean water for additional period of three months and six months did not indicate that the compound is carcinogenic to the fishes.

B. Materials and methods

Details of methods and procedures for small fish culture, range-finding tests, the exposure apparatus, exposure protocols, analytical chemistry, grow-out, and histological analyses are have been previously reported (Walker et al., 1986; Hawkins et al., 1988).

For the medaka tests, three hundred 6 day old fry (mean wet and dry weights of 3.34 and 0.53 mg/fry, respectively, based on a random sample of 50 similarly aged fry) were utilized. For the guppy tests, 300 fry (wet and dry weights of groups used for individual treatments ranged from 8.87-9.0 mg/fish and 1.67-2.46 mg/fish, respectively) with exception of the aquarium control group which received only 260 guppies because of the low production for that particular exposure. Guppies were less than or equal to 48-hours postpartuition when introduced into the treatment groups.

Test specimens were allotted to the following treatment groups.

(1) Aquarium control group (situated outside the exposure system)

(2) Flow-through control group (situated inside the exposure system and thus subject to low levels of volatile test compounds)

(3) Low concentration exposure group (continuous TeCE exposure for 90 days)

(4) Intermediate concentration exposure group (intermittent TeCE exposure administered once weekly for 24 hours throughout the 90 day exposure period)

(5) High concentration exposure group (intermittent TeCE exposure administered once weekly for 24 hours throughout the 90 day exposure period)

About 100 specimens from each treatment group were sampled for histopathological examination at 24, 36, and 52 weeks post-initial exposure. Details of histological procedures and criteria for lesion diagnoses have been published earlier (Hawkins et al. 1989a, 1989b).

C. Results

TeCE concentrations measured by electron-capture gas chromatography. Average TeCE concentrations for the treatment groups for the two species are shown below in Table IIa.

Table IIa. Average concentrations of 1,1,2,2-tetrachloroethane (TeCE) in treatment groups of medaka and guppy in 90-day carcinogenesis bioassays.

Treatment group	TeCE Concentrations Medaka	Guppy
Aquarium control	Not detected	Not detected
Flow-through control	0.024+/-0.015 mg/l	0.030+/-0.017 mg/l
Low concentration	3.970+/-1.350 mg/l	3.450+/-1.090 mg/l
Intermediate Concentration	7.760+/-0.350 mg/l	6.930+/-0.450 mg/l
High concentration	13.93+/-1.260 mg/l	12.780+/-1.30 mg/l

With both species, neither exposure nor grow-out mortality were dose-related and more that 92% of each species from each treatment group survived to grow-out. Histological examination of three whole specimens of each species from each treatment group taken at the end of the 90-day exposure did not reveal any toxicant-related pathological effects.

The results of histopathological examination of medaka exposed to TeCE are summarized in Table IIb with incidences of combined hepatic neoplastic lesions and brief descriptions of the lesions given. Based on this analysis, TeCE did not appear to be carcinogenic to the medaka.

Table IIb. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in Japanese medaka (Oryzias latipes) exposed to tetrachloroethane (TeCE). (FT 60 results as of 11-06-89)

Exposure Group	24 wk	36 wk	52 wk
Aq ctl	0/73	1/71	NE
Ft ctl	1?/72	NE	NE
4.0 TeCE	NE	NE	NE
Int. 8.0 TeCE	0/42	NE	NE
Int. 15.0 TeCE	0/75	1/74	1*/102

*indicates a cholangiocellular lesion
NE= Not Examined

Significant lesions in FT 60

Aq ctl	36wk	10318	I	adenoma
Ft ctl	24wk	9870	N	adenoma (clear cell variant-possibly not neoplastic)
		9872	K	lymphoma
15 TeCE	36wk	10313	M	adenoma
	52wk	11080	C	possible hepatocellular carcinoma with metastasis to pericardium
		11080	M	cholangiocarcinoma/gall bladder carcinoma

Similarly, the TeCE did not appear carcinogenic to the guppy. Those results are summarized in Table IIc. Because significant incidences of neoplasms were not seen in the high exposure group, only one control group or group exposed to lower TeCE concentrations was examined.

Table IIc. Incidences of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the guppy (Poecilia reticulata) exposed to tetrachloroethane (TeCE). (FT61 results as of 10-30-89)

Exposure Group	24 wk	36 wk	52 wk
Aq ctl	NE	1/74	NE
Ft ctl	NE	NE	NE
4.0 TeCE	NE	NE	NE
Int. 8.0 TeCE	NE	NE	NE
Int. 15.0 TeCE	0/76	0/75	2/97

NE= Not Examined

D. Discussion

The present study shows that 1,1,2,2-tetrachloroethane (TeCE) is not carcinogenic to medaka and guppy when administered in flow-through exposures for 90 days with specimens examined up to 52 weeks post-initial exposure. There are several ways we could interpret this negative result. The specimens might not have been exposed long enough for the compound to elicit a carcinogenic response. This is unlikely in the present study because three months represents a considerable portion of the life span of the medaka and guppy. Furthermore, the high concentration exposure, at least, was administered at near the toxic limit.

Possibly, TeCE was not carcinogenic to the medaka and the

guppy because those fishes are not capable of metabolizing the compound to its carcinogenic intermediates. TeCE has been shown to be hepatocarcinogenic in only one bioassay model, the B6C3F1 mouse, which is known to have a high spontaneous background of liver tumors (see Bolt, 1987). Because TeCE is considered highly hepatotoxic, at least to mammals, it is likely that recurrent hepatic damage and cell replication could contribute to its carcinogenicity. Generally, chlorinated solvents are not as hepatotoxic in fish as they are in mammals. It also appears that hepatic microsomal metabolism by cytochrome P450-dependent mixed function oxidases plays a role in the carcinogenicity of TeCE as well as other chloroethanes (Ivanetich and van den Honert, 1981). Because numerous studies have shown that fish are incapable of metabolizing compounds that require cytochrome P450, phenobarbital-like compounds, it may be that the medaka and guppy were not able to convert TeCE to its carcinogenic intermediates because of those deficiencies.

III. Carcinogenesis bioassay with the heavy metal cadmium on the Japanese medaka (Oryzias latipes)

A. Introduction

Cadmium is distributed widely in nature and affects man through occupational exposures mainly in smelters, through food consumption mainly in the form of contaminated seafood, and through tobacco use (Kazantzis, 1987). Absorbed cadmium is eventually bound to a low molecular weight metal binding protein, metallothionein. Metallothionein-bound cadmium accumulates mainly in the kidney proximal tubular cells.

Cadmium is associated with unusual patterns of carcinogenesis. Following subcutaneous injection in rats, it induces injection-site sarcomas (mainly fibrosarcomas and rhabdomyosarcomas) as well as reproductive toxicity and neoplasms in males (Haddow et al., 1961; Kazantzis and Hanbury, 1966; Lucis et al., 1972). Cadmium administered in drinking water, in the diet, or by gavage, however, did not increase tumor rates in rats (Loser, 1980).

We conducted cadmium exposures with Japanese medaka in which the fish were exposed for various periods of time to waterborne cadmium at near toxic levels. Our rationale was that this type of exposure would combine skin contact exposure, epithelial (gill) uptake exposure, and possible enteric exposure through consumption of the cadmium-containing water. Although cadmium exposure was not associated with increased carcinogenesis in medaka in this bioassay and there are gaps in the study, the study is presented here in detail to compare these negative results with studies involving other metals, fish species, and methods of exposure. We propose that the study be repeated using intraperitoneal

injection as the route of exposure.

B. Materials and Methods

Three hundred 6 to 7 day old medaka (mean wet and dry weights 2.60 and 0.48mg/fry, respectively, based on a random sample of fifty 6 day old fry) were assigned to each of the following treatment groups:

1. Nominal 30 ppb Cd, 1 x 6 hours
2. Nominal 30 ppb Cd, 1 x 12 hours
3. Nominal 30 ppb Cd, 1 x 24 hours
4. Nominal 30 ppb Cd, 2 x 24 hours, one week intervals
5. Nominal 30 ppb Cd, 3 x 24 hours, one week intervals
6. Nominal 30 ppb Cd, 4 x 24 hours, one week intervals

Each of these groups were matched with control groups which were handled identically to their cadmium-exposed counterparts. Preliminary experiments revealed that cadmium chloride prepared in distilled water and added to well water normally used in freshwater bioassay studies precipitated, presumably due to a combination of high alkalinity (240 mg/l calcium carbonate) and low hardness (1.8 mg/l calcium carbonate) in our well water. Therefore, we used a "synthetic" water that contained 96.0 mg $NaHCO_3$, 60.0 mg $CaSO_4.2H_2O$, 60 mg $MgSO_4$, and 4.0 mg KCl per liter of distilled water. Following the last exposure, specimens were removed to grow-out aquaria until sampled for histological examination as described in Section II.

C. Results and Discussion

Histopathological studies to date have not revealed a carcinogenic response in cadmium exposure medaka (Table IIIa.)

Table IIIa. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the Japanese medaka (Oryzias latipes) exposed to Cadmium.
(10-30-89)

Exposure Group	24 wk	36 wk	54 wk
Syn Ctl 24 hrX4	0/48	1/34	NE
Cd++ 24 hrX3	NE	NE	0/25
Cd++ 24 hrX4	0/75	0/58	NE

NE=Not Examined

Although we paid special attention to the possible development of hepatocellular neoplastic lesions, neoplastic lesions did not appear to develop in any other organs either. The absence of any kind of neoplastic response concerns us and we can apply the same analysis to this study that we did with the 1,1,2,2-tetrachloroethane (TeCE) study described above which also yielded negative results. In contrast with TeCE carcinogenesis in mammals which is highly species specific (see above), there appears to be little species-related sensitivity related to cadmium carcinogenesis in mammalian models. Given that there is little understanding of the carcinogenic mechanism of any metal including cadmium (Furst, 1987), it seems unlikely that the medaka would not be sensitive because of some species-related factor. Furthermore, because exposure concentrations were near the toxic levels to the medaka, it is unlikely that a carcinogenic exposure concentration was not reached. Before we assert that cadmium is not carcinogenic to small fish, we plan to repeat the tests using multiple intraperitoneal injections to deliver cadmium chloride to adult specimens of both the medaka and guppy.

IV. Studies on the carcinogenicity of the aromatic amine (2-acetylaminofluorene) in the Japanese medaka (Oryzias latipes) and king cobra guppy (Poecilia reticulata) and its metabolism in the medaka.

A. Introduction

The aromatic amines are a class of chemicals that include the carcinogens benzidine and aniline as well as 2-acetylaminofluorene (2-acetamidofluorene; N-2-fluorenylacetamide;2-AAF). Although the carcinogenicity of 2-AAF in rodents is well-known and it is widely used as a model carcinogen in initiation-promotion tests, its carcinogenicity, or the carcinogenicity of any other aromatic amine, has not been reported. For 2-AAF to be carcinogenic, it must be N-hydroxylated by a cytochrome P-450-dependent, microsomal-bound enzyme. Ring hydroxylation, on the other hand, by another P-450 enzyme appears to be a detoxification step.

B. Materials and Methods

Hepatic metabolic studies with 2-AAF were conducted on the Japanese medaka (Oryzias latipes). Medaka were at least three months old at the time of exposure. An acute, static exposure of AAF to medaka was conducted at the Gulf Coast Research Laboratory. Six 4-L flasks of a nominal concentration of 10 ppm AAF in well water was stirred in the dark for four days. Each was then filtered through a 0.2 micron Nucleopore filter and all filtrates added to an equal volume of well water in the exposure aquarium. The control aquarium contained unamended well water. Immediately prior to addition of the fish, duplicate water samples were

collected for quantitation of AAF concentration. Aquaria were held in the dark throughout the 48- to 72- hour exposure period. Test and control fish were occasionally examined under subdued light, and fish were not fed during the exposure. AAF-exposed fish were transported to the C.V. Whitney Laboratory, St. Augustine, Florida, for the biochemical analyses.

Livers were removed, weighed, and homogenized in ice cold 1.15% KCl, 0.02M HEPES buffer (pH 7.4) adjusted with the protease inhibitor phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 13,000 x g at 0 degrees C for 20 min, the pellet discarded, and the resulting supernatant centrifuged at 176,000 x g at 0 degrees C for 45 min to produce cytosolic and microsomal fractions. The microsomal fractions of control and AAF-exposed specimens were incubated with [9-14C]AAF according to Juchau et al. (1975) to search for metabolite profiles. Metabolites were identified by thin-layer chromatographic techniques using silica gel 150A TLC plates of 250 micron thickness with preadsorbent spotting area. Standards run simultaneously included N-hydroxy-2-AAF, 1-hydrdoxy-2-AAF, 3-hydroxy-2-AAF, 5-hydroxy-2-AAf, and 7-hydroxy-2-AAF. Another aliquot of each microsomal fraction was incubated with unlabelled N-OH-AAF and UDP-glucuronyl transferase activity was assayed using radiolabelled cofactors. The glucuride produced form this enzyme is a weakly reactive electrophile that is excreted in urine. In this way, the medaka's ability to detoxify the proximate carcinogen was assessed. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (1970) using a 4% acrylamide stacking gel and a 7.5% acrylamide separating gel introducing 20 ug microsomal protein per well. Epoxide hydrolase with styrene oxide (Oesch et al., 1971) was assayed in the microsomal fraction of both control and AAF-exposed fish. Preliminary tests produced a slight induction in a protein of approximately 49 KDalton molecular weight upon exposure to AAF. This is within the weight range of epoxide hydrolase enzymes. Therefore, the appearance of any novel protein bands in the gel was compared with epoxide hydrolase activity. Protein content was determined using the Folin and Ciocalteu's phenol reagent methods according to Lowry (1951).

For the carcinogenesis studies, medaka and guppy (Poecilia reticulata) were exposed to AAF by two mechanisms: (1) a static single pulse exposure and multiple intermittent pulse exposures; and, (2) under prolonged static-renewal conditions. All exposures were performed in the dark or subdued light. Treatment groups were as follows:

(1) Pulse exposures
   a. 1 x 6 hours
   b. 1 x 12 hours
   c. 2 x 12 hours, one week intervals
   d. 3 x 12 hours, one week intervals
   e. 4 x 12 hours, one week intervals

(2) Static renewal exposures

One group was exposed continuously for 168 hours (7 days) with renewal of AAF and control solutions every 24 hours.

Appropriate controls were included for each treatment group. Pulse exposures were conducted in one control aquarium and one AAF treatment aquarium. Medaka and guppies were exposed simultaneously in the same treatment aquarium with specimens for each treatment contained in individual mesh chambers. For each weekly pulse exposure, AAF was added to well water to produce a 10 mg/l nominal concentration, the mixture stirred in the dark at room temperature for 4 days, filtered through a 0.2 micron Nuclepore membrane filter, and the resulting suspension diluted 1:1 with well water and transferred to the exposure aquarium. Mesh chambers containing fish were added and a sample was taken at time zero (T-0) for analysis. The control aquarium contained unamended well(diluent) water. Water samples were also taken at 6 hours (T-6) and 12 hours (T-12) when individual treatments were terminated. Each treatment consisted of 300 specimens of 6-day post-hatch medaka (wet and dry weights 3.47 and 0.57 mg/fry, respectively) less than or equal to 48-hour post-parturition guppies (wet and dry weights means of groups 8.91 ± 0.63 and 1.86 ± 0.19 mg/fry, respectively). One large initial pool of medaka fry was used for all treatments whereas each guppy group came from a set of fry collected weekly. After the appropriate length and number of exposures, all fish were rinsed three times in well water, and the fish counted and placed in grow-out aquaria. In the static renewal test, both species were exposed simultaneously with guppies and medaka sequestered in mesh chambers in individual 4-l beakers. The AAF stock solution was prepared as described for the pulse exposure. Four 4-l beakers were used, 1 for control and 1 for AAF for each of the two species. Total volume in each beaker was 3-l with daily replacement being accomplished with a single 4-l AAF/well water preparation. A time zero-hour water sample was taken immediately after the mesh chamber containing fish was introduced into the exposure aquarium. The 24-hour sample was taken the following day before the mesh chamber was removed to a new 4-l beaker containing fresh solution (well water or AAF). Then, another time zero hour sample was taken. At the end of the 7 days exposure the fish were rinsed, counted and placed in grow-out aquaria. Concentrations in both types of exposures were around 1.0 mg/l.

Because there was no published methodology for the measurement of AAF in water, some preliminary studies were necessary. The aromatic ring structure of AAF suggested that AAF could be measured by fluorescence spectrophotometry which would permit direct analysis without prior extraction. However, the intensity of fluorescence of AAF in water solution was insufficient for the sensitivity required in this procedure.

Efficiency of extraction from a water medium using various solvents have slightly different polarities was then determined. Hexane, benzene, and dichloromethane were used to extract saturated solutions of AAF from distilled water. Dicholoromethane was determined to be the most efficient solvent for extraction. Gas chromatographic analysis using a flame ionization detector was employed and various column conditions were tested to find the best conditions for measurement of AAF. Methodology was finalized and monitoring was performed using a Perkin-Elmer 3920 gas chromatograph 15 m x 0.25 mm (i.d.) fussed silica glass capillary column with 0.25 micron coating of DB-1 (J&W Scientific). Quantitation was achieved by internal standard method using a Perkin-Elmer Sigma 10 Data System. Histopathological evaluations followed protocols described above.

C. Results

Biochemistry. To investigate the hepatic bioactivation of 2-AAF in medaka, a preliminary 48-hour exposure to 8.6 ppm 2-AAF was run and the activities of a series of mixed function oxidase enzymes (MFO's) assayed. The activity of the MFO ethoxycoumarin O-deethylase was suppressed by 2-AAF exposure and a new protein band at approximately 49 KDaltons was observed in the electrophoretic separation of the microsomal fraction of AAF-exposed fish as compared to controls. There was no difference in the bands of the cytosolic fractions of both treatments. For the definitive study, approximately 100 adult medaka were exposed to 2.1 ppm 2-AAF for 48 hours with 100 untreated medaka serving as controls. Exposed medaka were transferred to a dilute solution of 2-AAF for 24 hours during which time they were transported to the Whitney Laboratory where they were sacrificed. Incubation of 2-AAF with the microsomal fractions of both treatments resulted in a combined four-fold increase in the amount of combined 2-AAF metabolites in the control treatment compared with that in the 2-AAF pretreated microsomes (Table IVa). The major AAF metabolite formed in vitro was 7-OH-AAF, followed by 5-OH-AAF, both of which indicate ring hydroxylation. The carcinogenic intermediate, N-OH-AAF, was also produced demonstrating, at least qualitatively, the activation capability for aromatic amines of medaka hepatic enzyme systems. In summary, 2-AAF depresses hepatic microsomal oxidative enzyme activities whereas it increases glutathione S-transferase activity. Exposure to 2-AAF does not seem to affect the activities of epoxide hydrolase or of glucuronyl transferase. Medaka unexposed to 2-AAF appear to be able to hydroxylate AAF in vitro to mainly ring metabolites and to a lesser extent to the N-metabolite, N-hydroxy-AAF, the proximate carcinogen.

Histopathology. In specimens examined to date, it appears that AAF causes neoplastic lesions in livers of the guppy but not in the medaka. The incidences of those lesions are shown in Tables IVb and IVc for the medaka and guppy, respectively. The response in the two highest exposure levels in the guppy tests

were statistically significant by Fisher's Exact Test. No hepatic neoplastic lesions considered malignant, however, were diagnosed in the guppy. Most of the lesions were either foci of cellular alteration (altered foci) or were adenomas. None were diagnosed as hepatocellular carcinomas (Table IVc).

Table IVa. Biotransformation pathways in control and AAF-treated medaka.

Parameter measured	Control	Treated
Protein Yield, Microsomes	9.64	15.4
mg/g liver Cytosol	52.00	44.4
Oxygenation of AAF		
Total, pmole/min/mg protein	256	68.5
7-OH	210	48.4
5-OH	20	8.8
3-OH	4	1.9
1-OH	2	1.3
N-OH	5	0.7
Glucuronyl Transferase, pmole/min/mg/protein		
4-Methyl umbelliferone	556	680
3-Hydroxy AAF	181.2	244.6
Epoxide Hydrolase, nmole/min/mg protein	1.51	1.11
GSH-S-Transferase, nmole/min/mg protein	1370	1814
Sulfotransferase, pmole/min/mg protein		
4-Methyl umbelliferone	129.6	40
3-Hydroxy AAF	25.2	30.0
N-Hydroxy AAF*	184	75

Table IVb. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the Japanese medaka (Oryzias latipes) exposed to AAF. (10-30-89)

Exposure Group	24 wk	36 wk
Ctl 12 hrX4	0/53	0/74
Constant Ctl	0/66	NE
AAF 6hx1	0/77	NE
AAF 12hx1	0/50	NE
AAF 12hx2	2/51	NE
AAF 12 hrX4	0/52	0/74
AAF Constant (7 d)	3/63	0/76

Table IVc. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the guppy (Poecilia reticulata) exposed to AAF. (10-30-89)

Exposure Group	24 wk	36 wk
1. Ctl 12 hrX4	0/31	1/70
2. Constant Ctl	0/84	0/69
3. AAF 6 hrX3	NE	2/18
4. AAF 12 hrx1	NE	0/70
5. AAF 12 hrX4	3/98	7/73*
6. AAF Constant (7 d)	3/80	7/76*

NE- Not Examined

*Statistically significant at $p < 0.01$ when compared with controls by Fisher's Exact Test

Table IVd. Hepatic neoplastic lesions in guppy/ AAF exposure

Exposure group	Sampling period	Specimen No.	Lesion
1. Ctl 12hx4	36wk	11276 P	altered focus
3. AAF 6hx3	36wk	11309 O	adenoma
		11309 P	adenoma/carcinoma
5. AAF 12hx4	24wk	10694 K'	altered focus
		10694 K"	altered focus
		10692 J"	altered focus
	36wk	11265 H	adenoma
		11266 P'	altered focus
		11266 U	adenoma
		11267 N'	altered focus
		11267 O'	adenoma
		11267 Q	altered focus
		11267 T	adenoma
6. AAF Cnst (7 days)	24wk	10727 D	altered focus
		10727 L	altered focus
	36wk	11271 M	altered focus
		11272 L	adenoma
		11272 T'	altered focus
		11273 D	adenoma
		11273 K	adenoma
		11273 L	adenoma
		11273 N'	adenoma

D. Discussion

To our knowledge, studies on the hepatic metabolism of AAF in the medaka represent the first time that the relative refractoriness of a fish species to the carcinogenic effects of AAF has been related to the fish's inability to adequate metabolize the compound to is proximate carcinogen by N-hydroxylation relative to its ability to detoxify the compound by ring hydroxylation.

AAF caused a statistically significant increase in combined hepatic neoplastic lesions in the guppy. The low carcinogenic potency of this compound, however, was evidenced by the fact that many of the induced lesions, in spite of the fact that they persisted for 6 and 9 months postexposure, did not appear robust or actively progressing to more aggressive lesions. This is the first time in our studies that we have observed the apparent regression of carcinogen-induced lesions, even ones such as those we designate as altered foci which are generally terminal lesions in rodents. This poses some interesting questions regarding the

nature of neoplastic initiation in small fish models. Perhaps the persistence of neoplastic lesions is related to the dose x time or to the strength of the test carcinogen. To test whether species-specific differences in hepatic metabolism are responsible for the sensitivity, or resistance, to the carcinogenic effects of AAF we will conduct a simultaneous comparison of the metabolism of AAF in both the medaka and guppy. It would also be interesting to compare the carcinogenic effects of the proximate carcinogen, N-OH-AAF, on both species.

V. The occurrence of thymic lymphoma in carcinogenesis bioassay specimens of the Japanese medaka (Oryzias latipes)

A. Introduction

Neoplasms of hematopoietic origin have been reported in several species of fish. Of these, neoplasms of the lymphoid tissues have been the most frequently reported. Epizootics of lymphosarcoma have been reported in muskellunge, Esox masquinongy (Sonstegard 1975), and in northern pike (Esox lucius) of North America, Ireland, Sweden, and Finland (see Thompson 1982). Lymphosarcoma has been reported in Atlantic salmon (Salmo salar) (Roald and Hastein 1979) and rainbow trout (Salmo gairdneri) (Warr et al., 1984). Two cases of lymphosarcoma have been reported in N-methyl-N'-Nitro-N-nitrosoguanidine exposed channel catfish (Ictalurus punctatus) (Chen, Brittelli, and Muska 1985) and in dimethylbenz(a)anthracene exposed fish of the genus Poeciliopsis (Shultz and Shultz 1982). One case of lymphosarcoma has been reported in N-methyl-N-nitrosourea treated platyfish/swordtail hybrids (Schwab et al. 1978). In addition to this, lymphoma has been reported in cultured turbot (Scophthalmus maximus) (Ferguson and Roberts 1976).

The present report describes 40 cases of lymphoma in the Japanese medaka (Oryzias latipes) from carcinogenesis studies using this species. Twenty eight cases occurred in chemically treated groups and 12 cases occurred in control specimens.

B. Materials and Methods

Medaka approximately 6-10 days old were exposed to chemical carcinogens for one to 90 days. Following exposures the fish were transferred to aquaria containing carcinogen-free water. Routine samples for histopathological examination were taken at 24, 36, and 52 weeks. Moribund fish exhibiting a swelling of the head above or near the operculum were sampled as soon as they were identified. For light microscopy whole fish were fixed in Lillies fluid for 2-4 days, embedded in paraffin, sectioned at 5.0 μ, and were stained with hematoxylin and eosin. Moribund fish were treated in the same manner. Specimens for transmission electron microscopy (TEM) were cut from moribund fish exhibiting the tumor

on the head. The tissue was fixed in 3.0% glutaraldehyde in 0.1 M sodium cacodylate buffer and then post-fixed in 1% osmium tetroxide. Specimens were then dehydrated and embedded in Embed 812 resin. Thin sections were cut using a Reichert Ultracut E ultramicrotome. Grids were stained with uranyl acetate and lead citrate and examined with a JEOL 100 SX electron microscope.

C. Results

The cases of thymic lymphoma observed in our medaka cultures and carcinogenesis bioassays are listed in Table Va. In taking all cases as a whole, almost every tissue was infiltrated by sheets of lymphocytic cells. In early stages, lymphocytes appeared to infiltrate regions surrounding the thymus and gill arches. In more advanced stages lymphocytic cells infiltrated the orbit, cranium, musculature, kidney, visceral peritoneum, intestine, gonads, liver, and general circulation. The basophilic nucleus with a thin rim of cytoplasm was the prominent cellular feature. Mitotic figures were abundant. The lymphocytic cell appeared to be clustered into aggregates and did not show a linear arrangement which might have suggested a lining up along reticulin fibers.

The prominent feature seen by electron microscopic examination of the lymphoma cell was a deeply clefted nucleus. Nucleoli were also prominent in the lymphoma cell as compared with normal thymic lymphocytes. The thin rim of cytoplasm contained mainly free ribosomes, mitochondria, and frequently centrioles.

Table Va. Occurrence of Lymphosarcoma in the Japanese medaka

Test group	Sex	Age
DMF ctl	o	66 wk
Ctl	♂	28 wk
30% Puget Sound Microlayer A	♂	55 wk
	♂	55 wk
30% Puget Sound Microlayer B	♀	55 wk
DMBA/DMF/8μ	♂	40 wk
DMBA/DMF/10μ	♂	18 wk
Ctl	o	23 wk
DMF Ctl	♂	36 wk

Int BeP	o	51 wk
18 ppb DMBA	♀	34 wk
26 ppb DMBA	♀	38 wk
26 ppb DMBA	♀	52 wk
79 ppb DMBA	o	21 wk
150 ppb DMBA	♂	24 wk
Ft ctl	o	22 wk
10 DMBA/2 THM	♀	24 wk
Aq ctl	♂	52 wk
7.5 TCE/ 7.5 CCl_4	♀	35 wk
15 CDBM	♂	36 wk
0.1 EDB	♀	58 wk
1.0 EDB	o	31 wk
1.0 EDB	o	40 wk
1.0 EDB	♀	58 wk
20.0 EDB	o	20 wk
20.0 EDB	♂	35 wk
9.0 $CHCl_3$/$CHBr_3$/BDCM	♀	36 wk
Int. TBTO	o	41 wk
Aq Ctl	♀	36 wk
Ft Ctl	♂	24 wk
8.0 TeCE INT	♂	36 wk

DMF=dimethylformamide; CTL=control; DMBA=dimethylbenzanthracene;
BeP=benzo(e)pyrene; Ft ctl=flow-through control;
THM=trihalomethanes; Aq Ctl=aquarium control;
TCE=trichloroethylene; CC14=carbon tetrachloride;
CDBM=chlorodibromomethane; EDB=ethylene dibromide;
CHC13/CHBr3=chloroform/bromoform; TBTO=tributyltinoxide;
TeCE=tetrachloroethane_

D. Discussion

Carcinogenicity tests using small fish species have been found them to be responsive to a number of chemical carcinogens (Hawkins et al. 1985). Of several species of aquarium fish used so far, the Japanese medaka (Oryzias latipes) has been used to study the widest range of carcinogens and the pathology has been characterized more thoroughly (see Metcalfe, 1989). Data on spontaneous neoplastic lesions in this species should provide useful information for evaluating results in carcinogenesis studies.

By LM and TEM the cells resemble lymphoma in other species and the rainbow trout EM micrographs reported by Warr (et al., 1984). Viral particles were not observed in medaka lymphoma cells but that does not necessarily eliminate viruses in the etiology of the disease.

Comparing the medaka lymphoma with other cases that have been reported as lymphoma or as lymphosarcoma, it appears possible that the cases reported as lymphosarcoma in the channel catfish by Chen et al. (1985) and by Schultz and Schultz (1982) in Poeciliopsis, although they were reported in carcinogen treated fish are probably not carcinogen induced, but are showing some sort of reactive tissue.

In rodent bioassays it has been observed that there is a negative correlation between malignant lymphomas and hepatocellular proliferative lesions. A failure to recognize this trend may distort interpretation of carcinogenicity data for a particular test compound (Young and Gries, 1984). In our studies lymphosarcoma does not appear to affect the rate of hepatocellular lesions induced by carcinogens. This may be due to the comparatively low incidence of lymphosarcoma in medaka.

In summary, the medaka carcinogenesis bioassay provides a unique system in which to study thymic lymphoma with respect to its pathology, possible viral or chemical induction, and relationship with other neoplastic lesions.

VI. The occurrence of acinar cell carcinomas of the exocrine pancreas in the Japanese medaka (Oryzias latipes)

A. Introduction

Exocrine pancreatic neoplasms are uncommon in fishes occurring mainly as isolated cases in diverse species (Fournie and Hawkins, In Press). Chemical induction of acinar cell exocrine pancreatic neoplasms has been reported in guppies Poecilia reticulata exposed to methylazoxymethanol acetate (MAM), a direct-acting carcinogen (Fournie et al., 1987) and in a

single specimen of the bullminnow Fundulus grandis that had been injected with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as an embryo (Grizzle et al., 1988). Thiyagarajah and Grizzle (1986) described pancreatic neoplasms of duct cell origin induced by diethylnitrosamine (DEN) in rivulus Rivulus marmoratus. Pancreatic acinar cell metaplasia occurred in DEN-induced hepatocellular tumors in rainbow trout Salmo gairdneri (Lee et al., 1989).

The present report concerns the occurrence of exocrine pancreatic carcinomas in specimens of the Japanese medaka Oryzias latipes used in carcinogenesis bioassays. The medaka is exceptionally sensitive to the hepatocarcinogenic effects of several compounds including MAM, DEN, and various mycotoxins (see review by Harshbarger and Couch, 1985) and to benzo(a)pyrene, 7,12-dimethylbenz(a)pyrene, and ethylene dibromide (Hawkins et al., 1989a,b,and unpublished). Furthermore, medaka are susceptible to the chemical induction of several types of extrahepatic neoplasms (Hawkins et al., 1986).

B. Materials and Methods

Specimens examined in this study came from carcinogenesis tests conducted in our laboratory since 1982. The tests were carried out generally as follows. Orange-red variety Japanese medaka (Oryzias latipes) approximately 6 to 10 days old were exposed to a variety of test chemicals for times that ranged from 1 hour to 180 days. Tests always included appropriate control groups. Following the exposures, fish were transferred to aquaria containing carcinogen-free water. Fish were fed a diet consisting of a commercially available flake food along with live artemia. Routinely, samples for histopathological examination were taken at 24, 36, and 52 weeks, measured from the beginning of the exposure. In other words, sampling times were approximately equal to the age of the specimens. Moribund specimens were also processed for histological examination. For light microscopy, whole fish were fixed in Lillie's fluid for 2 to 4 days, embedded in paraffin, sectioned at 5.0 um, and were stained with hematoxylin and eosin. Two specimens embedded in paraffin were reprocessed into epoxy resin for examination by transmission electron microscopy.

C. Results

Exocrine pancreatic carcinomas were diagnosed in eight specimens of the Japanese medaka Oryzias latipes out of approximately 10,000 used in a variety of carcinogenesis tests. Lesions occurred in the following cases: (1) a 44 week old female control specimen; (2) a 73 week old female control specimen; (3) a 24 week old female exposed to benzo(e)pyrene (BeP); (4) a 43 week old male exposed to (BeP); (5) a 33 week old female exposed to 7,12-dimethylbenz(a)anthracene (DMBA); (6) a 78 week old female exposed to DMBA; (7) a 42 week old male exposed to

benzo(a)pyrene; and, a 36 week old female exposed to methylazoxymethanol acetate (MAM). The neoplasm was probably the cause of death in each case except the 24 week old BeP specimen and the 36 week old MAM specimen which were from scheduled sacrifices. The neoplasms appeared to originate from an area near the abdominal surface of the liver and infiltrate the peritoneal cavity sometimes invading the gonads, intestine and kidney. In some cases, the disease became blood-borne as evidenced by the presence of tumor cells along trabeculae of the atrial myocardium. In one case, the tumor consisted of well-differentiated pancreatic acinar cells that invaded into the intestinal lamina propria. In the other cases, tumor cells were basophilic cells that were poorly differentiated, had a high nucleus to cytoplasm ratio, numerous mitoses, and tended to form cords and ducts or acini.

D. Discussion

There are several compelling reasons for studying the occurrence and biology of rare neoplasms in small fish carcinogenesis models. In developing carcinogenesis model systems, one needs to know as much as possible about the capability of the model to develop any kind of neoplastic lesion, whether spontaneous or chemically induce. Especially with studies that might require large numbers of specimens such as dose-response studies at low carcinogen exposures, associations between neoplastic lesions in primary target organs and those developing in secondary target organs, or, in non-target organs might be resolved.

The occurrence of spontaneous exocrine pancreatic neoplasms in medaka contrasts with the high sensitivity of the guppy to the pancreaticocarcinogenic effects of methylazoxymethanol acetate (Fournie et al., 1987. Under identical exposure conditions, neither medaka or five other small fish species developed exocrine pancreatic neoplasms although all developed hepatocellular ones (Hawkins et al., 1986; unpublished observations).

Based on the scattered occurrence of the disease, we consider it to be spontaneous and probably not induced by carcinogen exposure. Based on histology, behavior, and knowledge of the disease in other fishes, we consider the lesion to be acinar cell or, possibly, stem cell in origin.

VII. Studies on the hepatic metabolism of ethylene dibromide (1,2-dibromoethane) in the Japanese medaka

A. Introduction

Ethylene dibromide (1,2-dibromoethane; EDB) is a halogenated aliphatic hydrocarbon that has been used as a pesticide and

gasoline additive and is of concern to humans because of potential industrial and environmental exposures (Brown, 1984; Hanson, 1984). In rodents, EDB induces neoplasms mainly at the site of exposure when administered chronically by gavage or inhalation (Weisburger, 1977; Olson et al, 1973; Wong et al, 1982). Neoplastic lesions of the liver have been induced by EDB exposure (Wong et al, 1982; Moslen, 1984) but the liver and other internal organs appear less sensitive than directly exposed tissues. EDB has an unusual carcinogenic mechanism that utilizes a detoxification pathway, conjugation with glutathione, to form an electrophilic compound that binds to DNA and initiates carcinogenesis (Guengerich et al., 1987).

A carcinogenesis bioassay with EDB has already shown it to be highly hepatocarcinogenic in the medaka (Unpublished observations). As part of this project, we began studies designed to elucidate the carcinogenic mechanisms of EDB in medaka. Here, we report the results of preliminary studies on glutathione S-transferase activities in EDB-exposed and control medaka.

B. Materials and Methods

Adult medaka were exposed to approximately 1.0 ppm EDB for 14 days under flow-through conditions then transported the C.V. Whitney Laboratory, St. Augustine, Florida, for biochemical analyses. Control and exposed specimens were sacrificed 24 hours after arrival. Livers from control and exposed medaka were pooled into three groups, weighed, homogenized, centrifuged, and treated as separate entities for statistical purposes. Glutathione S-transferase (GST) activities were determined according to James et al., (1976) in the cytosolic fractions. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (1970) using a 4% acrylamide stacking gel and a 7.5% acrylamide separating gel introducing 20 ug microsomal protein per well.

C. Results and Discussion

Analysis of glutathione S-transferase activities showed an induction of activity in EDB-exposed medaka (Table VIIa).

Table VIIa. Glutathione S-transferase activities in Japanese medaka exposed to 1.0 ppm EDB for 14 days. Activities expressed as nmole/min/mg protein

Liver pool	Control	EDB-exposed
1	5501	6353
2	5814	6708
3	3984	7475
Mean values	5100	6845
Standard deviation	979	573

Gel electrophoresis of the cytosolic proteins from medaka exposed to 1.3 ppm EDB for 14 days yielded a 26 kDalton protein band. These studies show that EDB exposure results in an increase in glutathione S-transferase activity that possibly results in an increase in the rate of conjugation of glutathione to EDB and in the amount of conjugated EDB available for binding to DNA. Other preliminary studies have indicated that EDB exposure in medaka suppresses the activities of hepatic microsomal mixed function oxidases that normally metabolize polycyclic aromatic hydrocarbons. Suppression by EDB of mixed function oxidases could have important implications in assessing the carcinogenicity of mixtures with the medaka model.

VIII. Conclusions

Although the project is only a little more than half over, we have made considerable progress toward our stated goal, namely to expand the usefulness of small fish carcinogenesis models and to enhance our understanding of the mechanisms and endpoints of their responsiveness to carcinogens. Carcinogenesis tests with TeCE and cadmium were negative. Possibly, the small fish are incapable of metabolizing some halogenated hydrocarbons to their carcinogenic intermediates. The cadmium test will be repeated using injection rather than water-borne exposure. Studies AAF yielded interesting results. AAF was not carcinogenic to medaka but was to the guppy. Metabolic studies performed on the medaka suggest that it is more efficient in detoxifying AAF than it is in producing the carcinogenic metabolites. We now plan to examine the metabolism of AAF in the guppy and compare it with the medaka. Studies on the hepatic metabolism of EDB in medaka showed that it induces the phase II enzyme glutathione S-transferase which is involved in production of the ultimate carcinogenic species of EDB. Studies

on thymic lymphoma and acinar cell carcinomas of the exocrine pancreas in medaka focussed on understanding the pathology and progression of these lesions and, as relatively rarely occurring tumors, how they would affect the interpretation of large scale carcinogenesis bioassays with small fish.

Other tests planned or already underway include carcinogenesis bioassays with the following compounds: (1) vinylidene chloride, a potentially carcinogenic halogenated hydrocarbon; (2) chlorodibromomethane, an hepatic toxicant that might elicit a carcinogenic through a damage/repair mechanism; (3) methapyrilene, an antihistamine that is hepatocarcinogenic in rats; and, (4) dibenzocarbazole, a nitrogen heterocyclic that is an environmental contaminant to both man and wildlife. The activation and detoxification of AAF in the guppy will be examined simultaneously with repeat studies on the medaka. We will also continue analyses of non-hepatic neoplastic lesions in small fish carcinogenesis.

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