Chapter 19

Chronic Inflammatory Lesions in Two Small Fish Species, Medaka (*Oryzias latipes*) and Guppy (*Poecilia reticulata*), Used in Carcinogenesis Bioassays

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ABSTRACT

Chronic inflammatory lesions affect two laboratory-reared fish species, the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*) that are used as carcinogen bioassay models. The present study investigated some aspects of the biology of the inflammatory lesions and their relationships to responses by the medaka and guppy to two chemical carcinogens. A total of 5093 fish specimens from bioassays with 7,12-dimethylbenz[*a*]anthracene (DMBA) and 2-acetylaminofluorene (AAF) were examined histologically to determine the prevalence, tissue distribution and morphology of chronic inflammatory lesions, and their relationship to responses to carcinogen exposure. Whereas the medaka typically exhibited a diffuse inflammatory response usually in the kidney, the guppy displayed morphologically diverse nodular lesions usually in the liver. Giant cells occurred frequently in kidney lesions of the medaka but not in the guppy. The occurrence of acid fast bacteria (AFB) was examined by Ziehl-Neelsen (ZN) staining of representative specimens of the two species from the DMBA study. Intact AFB were demonstrated in lesions in the guppy and ZN-positive debris in the medaka. About 40% of the cases in the guppy were associated with AFB, presumably a naturally-occurring mycobacterial infection. For each guppy and medaka with an inflammatory lesion, a degree of severity was determined and regression analysis (RA) used to examine factors that most influenced severity ratings. RA indicated that severity was higher in guppies, in older specimens of both species, in specimens exhibiting diffuse inflammatory reactions, and in AFB-positive cases. The results suggested that the inflammatory lesions occurred independently of neoplastic responses.

INTRODUCTION

The application of small laboratory fish species such as the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*) as carcinogenesis models requires understanding of both the non-neoplastic and neoplastic lesions in the species. The occurrence of chronic inflammatory or granulomatous lesions is a major concern. Such lesions appear to affect most laboratory cultures of medaka and guppies, especially older specimens. Because of disagreements regarding the classification of cells comprising the lesions in fish, it has been suggested that they be referred to as chronic inflammatory foci rather than granuloma, the traditional term (Noga *et al.*, 1989, 1990).

Chronic inflammatory lesions frequently, but not always, have an infectious etiology. For example, in some human cases, the occurrence of granuloma may represent a host response to a neoplasm (Hollingsworth *et al.*, 1993). Whether or not chronic inflammatory lesions modify carcinogenic responses in fish is not known. Chronic inflammatory lesions may contribute to morbidity or mortality in fish by disrupting normal physiological processes (Smith and Gill, 1988). Following induction of chronic inflammatory lesions by an infectious agent, the effect on the immune system with regard to previous or subsequent carcinogenic stimuli is not known. Furthermore, because the inflammatory lesions are proliferative, there is the potential for misdiagnosis as a neoplasm (Harshbarger, 1984).

Bacteria, parasites, and fungi have all been implicated in the development of chronic inflammatory lesions in fish (Balouet and Baudin Laurencin, 1986). Among the bacterial agents, mycobacteria appear to be the most common cause of the lesions, especially those that develop into granuloma. Mycobacterial infection in fish is sometimes referred to as fish tuberculosis (Parisot and Wood, 1960), and described as a chronic systemic inflammatory disease that can affect any organ system frequently in aquarium-reared fishes (Van Duijn, 1981; Giavenni *et al.*, 1980; Leibovitz, 1980; Dulin, 1979).

The primary purpose of this study was to determine from histologic specimens the prevalence of chronic inflammatory lesions in medaka and guppies from tests that examined the carcinogenicity of 7, 12-dimethylbenz[α]anthracene (DMBA) and 2-acetylaminofluorene (AAF). We wished to determine whether the occurrence of the inflammatory lesions was induced or enhanced by carcinogen exposure. Also, the presence of acid-fast organisms, presumably mycobacteria in the inflammatory lesions, was determined by Ziehl-Neelsen staining of guppy and medaka tissue sections from the DMBA study.

MATERIALS AND METHODS

Fish

Histologic sections of medaka and guppy from bioassays of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 2-acetylaminofluorene (AAF) were examined. Because the lesions are considered to have an infectious etiology related to culture practices, culture conditions are presented in detail. Test medaka ultimately came from brood stock originally purchased in 1984 from Carolina Biological, Burlington, NC. They were maintained at a 3:2 female:male ratio with a loading density of about 1 fish per L in 40 L or larger aquaria containing well water that had been aged for at least 24 hr. Brood tanks were held at 25-28°C and provided a daily light regime of 16 hr light and 8 hr

dark. Fish were fed dry food (Prime Flakes, Zeigler Bros., Inc. or Stress Flakes, Aquavet)) sparingly 3 times daily and provided live brine shrimp (*Artemia* sp.) nauplii once daily. Feces and other debris were removed from the aquaria twice each week, with a concomitant water change of at least 20%. Aeration and continual water filtration were provided by biological sponge filters. Eggs were collected by siphoning them from the tank bottom, teasing them away from a netted female, or removing them from the sponge filters following egg deposition by the female. Individual eggs were placed in hatching solution in 4 L aerated jars at a density not exceeding 1 egg per mL. Temperature was maintained at 24°C. The hatching solution contained 100 mg NaCl, 3 mg KCl, 4 mg $CaCl_2.2H_2O$, and 16.3 mg $MgSO_4.7H_2O$ per 100 mL glass distilled water. Dead or diseased embryos were discarded. As fry hatched (about 10 day following fertilization), they were transferred to finger bowls containing approximately 1400 mL water with an average of forty fry per bowl and provided ciliates for 3 day and nematodes for 6 or more day. Bowls were maintained in a 27°C water bath. Feces and debris were removed daily with a concomitant 50% water change. After 3 day, newly hatched brine shrimp nauplii were added as a food source, and after 6 to 10 day fry were fed dry food and brine shrimp. Fry at or about 6 day age were transferred to beakers for testing or at about 15 day to maintenance aquaria for providing future brood stock. Water quality, temperature, and manipulations in maintenance aquaria were as described for brood aquaria with the exception of a 12 hr:12 hr light:dark cycle.

Initial guppy brood stock were purchased from Aqua World, Inc., St. Louis, Missouri. Brood guppies were maintained in 120 L aquaria at a loading density of approximately 1 fish per 2 L and a 3:5 male:female ratio. Most parameters were similar to those used for medaka. Newborn fry were afforded refuge in a thick floating vegetative mat of hornwort. Brood rooms were exposed to natural sunlight and enhanced by an artificial 12 hr light:12 hr dark photoperiod. During periods of fry collection, the photoperiod was increased to 16 hr light:8 hr dark. Brood fish were fed dry food three times daily and live brine shrimp nauplii once daily. In collection aquaria, feeding and grassed brood areas were divided by plexiglas strips to prevent food from entering protective vegetated areas. Fry were collected by netting and maintained at a density of 50-80 fry in 80 L aquaria filled with water from the brood aquaria. Fry aquaria were maintained under feeding, lighting, filtration, and temperature regimes identical to those in brood aquaria.

DMBA study

Tests using medaka and guppy incorporated two control groups and three DMBA treatment groups as follows: (1) an untreated control group, (2) a carrier control group exposed to 0.5 mL/L dimethylformamide (DMF), (3) a group exposed to a 0.45 µm filtrate of DMBA without DMF carrier (low concentration-about 0.5 ppm), (4) a group exposed to a 0.45 µm filtrate of DMBA with DMF carrier (intermediate concentration-about 20 ppm), and (5) a group exposed to a glass-fiber filtrate of DMBA with DMF carrier (high dose-about 50 ppm) (Hawkins *et al.*, 1989, 1990). The low concentration was prepared by adding granular DMBA (Aldrich Chemical Company, Milwaukee, WI) to 3 L of well water to produce a nominal concentration of 10 parts per million (ppm). This suspension was stirred in the dark for 3 or 4 day, filtered through a glass-fiber filter, then filtered again through a 0.45-µm Millipore® filter. A 200-mL portion of this filtrate was used for chemical analysis and the remaining 2,800 mL for exposure of the fish. Exposure media for the intermediate and high concentrations were prepared from a stock of DMBA dissolved in DMF. In both treatments where DMBA was dissolved in DMF, 3 L of well water were added to produce a nominal concentration of 10 ppm-DMBA. The intermediate dose was then prepared by passing the aqueous

DMBA-DMF suspension through a glass-fiber filter, then through a 0.45-µm filter. The high dose was prepared by passing the DMBA-DMF suspension through a glass-fiber filter only.

Exposures were carried out in 1L glass beakers in a carcinogen glove box (Labconco®) under static conditions, in the dark, at 26±1°C. Tests began with at least 145 specimens per test group. Specimens were 6-10 day old at initial exposure. Initial exposures lasted 6 hr after which the fish were removed to noncontaminated water for 6 day and then exposed three additional times for 6 hr each at intervals of 7 day. Fish were not fed during the 6-hr exposure periods. Following the fourth exposure, fish were moved to static growout aquaria containing noncontaminated water maintained at 26±1°C. Fish were fed dry flake food supplemented daily with live brine shrimp nauplii. Samples for histopathology were taken at 24 and 36 week post-initial exposure. Moribund specimens were also processed for histological examination as they were identified.

AAF study

Medaka and guppy were exposed to AAF by two mechanisms: (1) a single or multiple pulse exposure or (2) prolonged static-renewal exposure (Exposure media containing AFF was made up as described, James *et al.*, 1993). Exposures were static and were conducted in the dark or subdued light. Treatment groups for the pulse exposures included: 1 x 6 hr, 1 x 12 hr, 2 x 12 hr, 3 x 12 hr, and 4 x 12 hr. A 1 week interval separated the multiple (2, 3, and 4 x) exposures. For the static renewal exposures, treatment groups of medaka and guppies were exposed continuously for 168 hr (7 day) with renewal of AAF and control solutions every 24 hr. 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 separate mesh chambers. Concentrations of AAF in exposure media were determined by dichloromethane extraction of a sample of the filtered suspension, and gas chromatographic analysis of the extract with flame ionization detection.

Each treatment consisted of 300 specimens. Medaka were 6-day post hatch individuals. Guppies were less than or equal to 48 hr post-parturition. 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 exposure, specimens were rinsed three times in well water, counted and placed in grow-out aquaria and held until sampling for histopathology at 24, 36, or 52 week post initial exposure.

For the static renewal test, the AAF stock solution was prepared as described for the pulse exposure. Medaka and guppies were exposed simultaneously to AAF in two separate 4 L beakers. Two additional 4 L beakers maintained under conditions identical to the exposure systems but without AAF, served as controls. Total volume in each beaker was 3 L with daily replacement from a single AAF/well water preparation for exposed fish or well water-only preparations for controls. A time zero water sample was taken immediately after the mesh chamber containing fish was introduced into the exposure aquarium. A 24-hr sample was taken the following day before the mesh chamber was removed to a new beaker containing fresh solution (well water or AAF), at which time another time zero sample was taken. At the end of the 7-day exposure the fish were rinsed, counted and placed in grow-out aquaria. Concentrations of AAF in both types of exposures were about 1.0 mg/L.

Histopathological procedures

Whole fish specimens were narcotized in ice water or MS-222 (tricaine methanesulfonate), the abdominal cavities of larger specimens were slit open and whole fish placed into Lillie's fixative (10% formalin; 85% saturated aqueous solution of picric acid; 5% formic acid). Depending on the size, specimens were fixed for 24 hr to 1 week. Extended fixation times insured decalcification of the larger specimens. Specimens were then dehydrated in a graded series of ethanol, cleared in xylene substitute and embedded in paraffin. Specimens were embedded side down for the DMBA test and belly down for the AAF test. Sections were cut at 5 μm in two separate planes and stained with hematoxylin and eosin. Liver was the expected target organ in the original carcinogenesis studies but other tissues appearing in those planes of section were also routinely examined.

Histologic evaluation

Slides of whole fish specimens recorded as having chronic inflammatory lesions when initially screened for neoplastic lesions were re-evaluated histologically. The following organs and tissues, when present, were examined in each specimen: eye, gill, heart, liver, kidney, spleen, intestine, ovary, testis, and musculature. The organs affected and the morphology of the chronic inflammatory lesions were recorded. Also, the overall tissue damage imposed by the inflammatory lesions was assessed and assigned a severity rating on a scale of 1 (slight) to 4 (severe), based on the extent of dissemination (number of organs affected) and the amount of normal tissue displacement (number and diameter of inflammatory lesions within organs). A system for classifying and coding histological data similar to that proposed by Reimschuessel *et al.* (1992) was used for the histological evaluation and regression analysis components of this study.

Ziehl-Neelsen Staining

To determine if acid-fast bacteria (AFB) were associated with the inflammatory lesions, Ziehl-Neelsen (ZN) staining was done on specimens from the original DMBA study, each representing a plane of section between the two slides examined for the histologic evaluation in the present study. The major criterion for a positive classification was the presence of intact magenta colored, rod-shaped bacteria. Slides were initially screened on low power and if there was no evidence of magenta particles associated with the lesions, the specimen was considered negative. If magenta particles were seen on low power, the specimen was examined further under oil immersion (1000X). If only acid-fast debris was seen at 1000X and intact rods were not clearly visible, then the specimen was considered negative.

Chi-square test

Prevalence data were analyzed by chi-square to test for the independence of lesion occurrence among individual and between pooled test groups. Chi-square comparisons of prevalence data were intended to determine if chronic inflammatory lesions were induced by carcinogen exposure.

Regression analyses

A stepwise multiple regression analysis was used to identify variables which most influenced the severity rating in each of the two studies. The default stepwise procedure (PROC STEPWISE) of Statistical Analysis System (SAS, 1982) was used to generate the regression models. The variables

tested were species, sex, age, exposure (consisting of 5 variables in the DMBA study and 8 in the AAF study), and lesion morphology. Another variable, presence of acid-fast bacteria, was tested in the DMBA study. For lesion morphology to be included in the analyses, each specimen was placed in one of two categories: "exclusively nodular" or "otherwise." The "otherwise" category included any cases exhibiting a nonspecific inflammatory reaction in addition to or independent of nodular lesions. Nominal scale data were treated as indicator variables (Neter and Wasserman, 1974). Regression analysis was primarily intended to determine if severity ratings were higher with any particular exposure regimen although other variables were included.

RESULTS

Prevalence of chronic inflammatory lesions

Table 1 shows the prevalence of chronic inflammatory lesions in medaka and guppies from the DMBA study. Chi-square analyses revealed no significant differences among individual test groups (medaka χ^2= 7.490; guppy χ^2= 7.040, p <0.05) or between pooled control and treatment groups (medaka χ^2= 0.187; guppy χ^2= 2.028, p<0.05). However, there was a significantly lower prevalence of chronic inflammatory lesions in those groups that used DMF for both medaka (χ^2= 4.873, p <0.05) and guppy (χ^2= 5.149, p <0.05) when control and low dose groups which did not incorporate DMF were pooled and tested against pooled solvent (DMF) control, middle dose and high dose groups that incorporated DMF.

Table 1
Prevalence of chronic inflammatory lesions (CIL) in medaka and guppy exposed to 7, 12-dimethylbenz[*a*]anthracene (DMBA).

	Medaka		Guppy	
Test Group	No. fish examined	No. with CIL (%)	No. fish examined	No. with CIL (%)
Control	170	12 (7%)	139	16 (12%)
DMF control	167	8 (5%)	129	8 (6%)
Low DMBA dose	173	14 (8%)	131	11 (8%)
Middle DMBA dose	155	7 (5%)	133	8 (6%)
High DMBA dose	77	0 (0%)	124	4 (3%)

Table 2 shows the prevalence of chronic inflammatory lesions from the AAF study. Although there were significant differences for the medaka (X^2= 22.529, p <0.01) and guppy (X^2= 42.07, p< 0.01) in lesion prevalence among individual AAF test groups based on chi-square analyses, no outstanding pattern was identified. There was a significantly higher prevalence of lesions in the pooled controls compared to the pooled treatment groups for the medaka (X^2=9.395, p< 0.01) only.

Table 2
Prevalence of chronic inflammatory lesions (CIL) in medaka and guppy exposed to acetylaminofluorene (AAF).

	Medaka		Guppy	
Test Group	No. fish examined	No. with CIL (%)	No. fish examined	No. with CIL (%)
Control/ 12 hr x 4	226	7 (3%)	252	22 (8%)
Control/ Constant	254	20 (8%)	235	59 (25%)
AAF 6 hr x 1	258	3 (1%)	255	74 (29%)
AAF 12 hr x 1	222	10 (5%)	273	55 (20%)
AAF 12 hr x 2	230	5 (2%)	260	39 (15%)
AAF 12 hr x 3	33	0 (0%)	232	49 (21%)
AAF 12 hr x 4	229	5 (2%)	239	29 (12%)
AAF Constant	238	8 (3%)	259	45 (17%)

Histologic Evaluation

Approximately two-thirds of the DMBA-exposed medaka, usually moribund specimens sacrificed prior to a scheduled sampling date, exhibited a nonspecific inflammatory reaction, predominantly in the kidney (Figure 1). Hematopoietic tissue of the kidney was often replaced by inflammatory cells, sometimes to the extent that few urinary tubules remained. Giant cells were frequently present within the extensive kidney lesions (Figure 2). In specimens from the AAF study, the kidney was rarely observed because of the belly-down embedding protocol used. However, when the kidney was present, the inflammatory condition was usually present although giant cells were less apparent. Nodular lesions were rare in the medaka.

The guppy lesions were mainly nodular ones in various stages of development (Figure 3) and exhibiting considerable morphological diversity (Figure 4). The liver was the organ most often affected. Staining characteristics of the early, solid inflammatory lesions ranged from pale basophilic to light brown. The necrotic, nodular lesions of the guppy took on many forms that varied from round-to-ovoid to large and irregular in shape. Melanomacrophage centers with dark pigment deposits were frequently noted in guppies (Figure 5).

Ziehl-Neelsen Staining

No intact acid-fast bacteria (AFB) were observed in medaka specimens from the DMBA test although faintly stained acid-fast debris was sometimes present. Of 47 guppy specimens stained by the Ziehl-Neelsen method, 19 (40%) were positive for AFB. Intact acid-fast bacilli were predominantly found in solid inflammatory lesions (Figure 6) but occurred also in necrotic lesions.

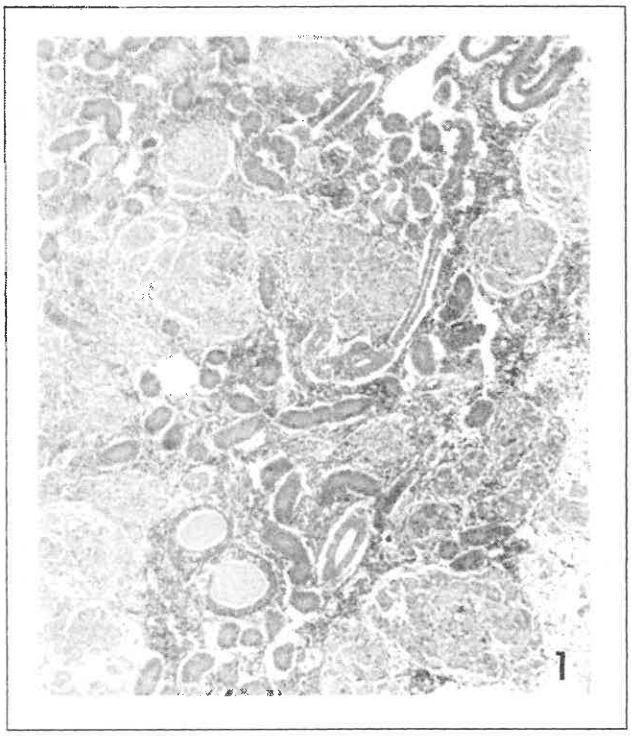

Figure 1. Medaka kidney with extensive inflammatory lesions. Control specimen, 57 week old. Hematoxylin and eosin.

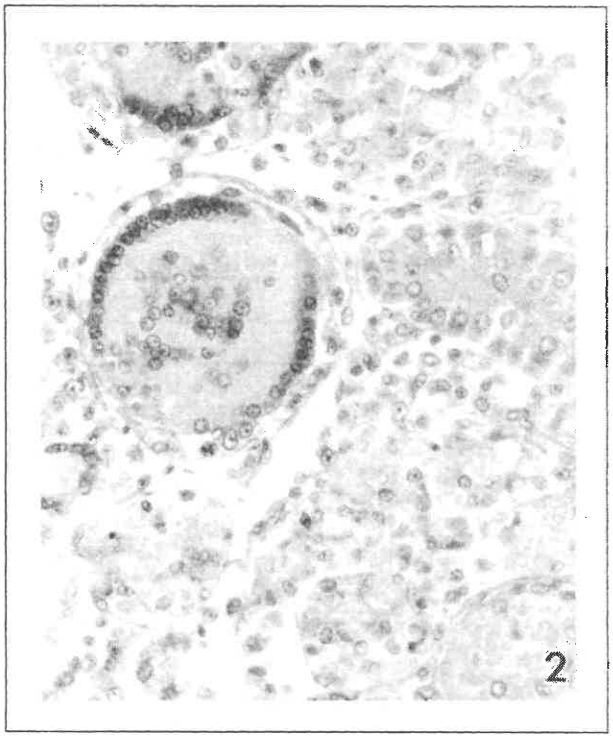

Figure 2. Multinuclear giant cell in kidney or medaka. DMBA exposed specimen, 55 week old. Hematoxylin and eosin.

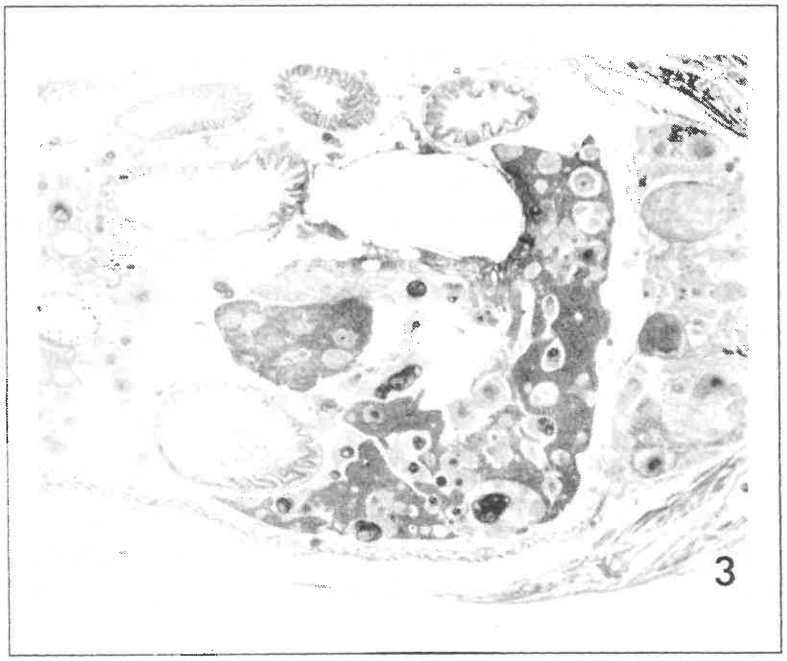

Figure 3. Widespread chronic inflammatory lesions in guppy. Note nodular lesions (from right to left) in cardiac region, liver, spleen and ovary. Specimen exposed to AFF, 29 week old. Hematoxylin and eosin.

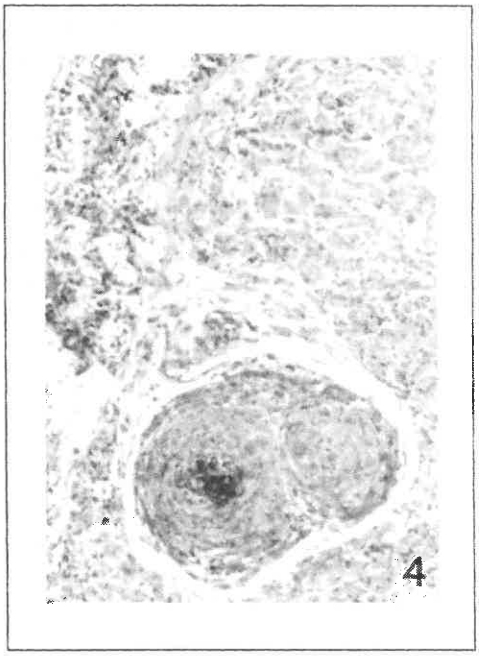

Figure 4. Diverse chronic inflammatory lesions in guppy kidney. Control specimen, 37 week old. Hematoxylin and eosin.

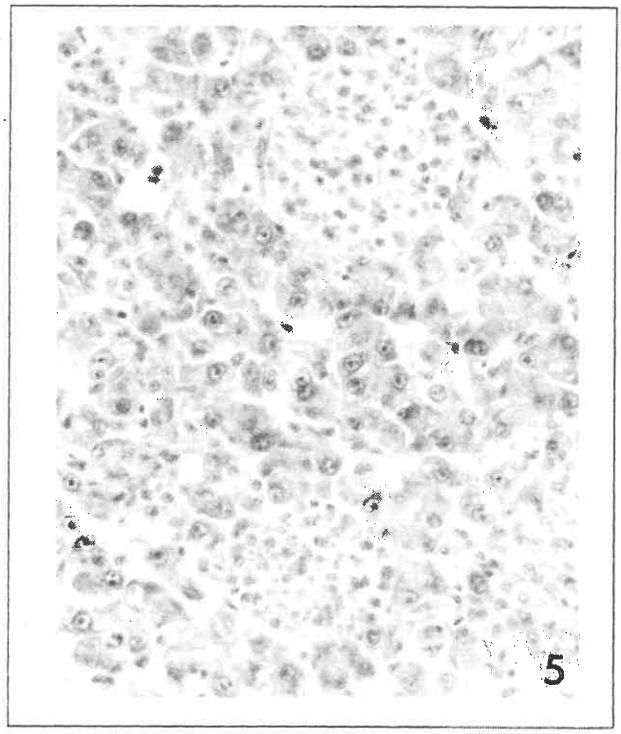

Figure 5. Two macrophage aggregates in the liver of a guppy. Specimen exposed to AAF, 52 weeks old. Hematoxylin and eosin.

Magnification:
Figure 1 = X70
Figure 2 = X480
Figure 3 = X15
Figure 4 = X200
Figure 5 = X480
Figure 6 = X480

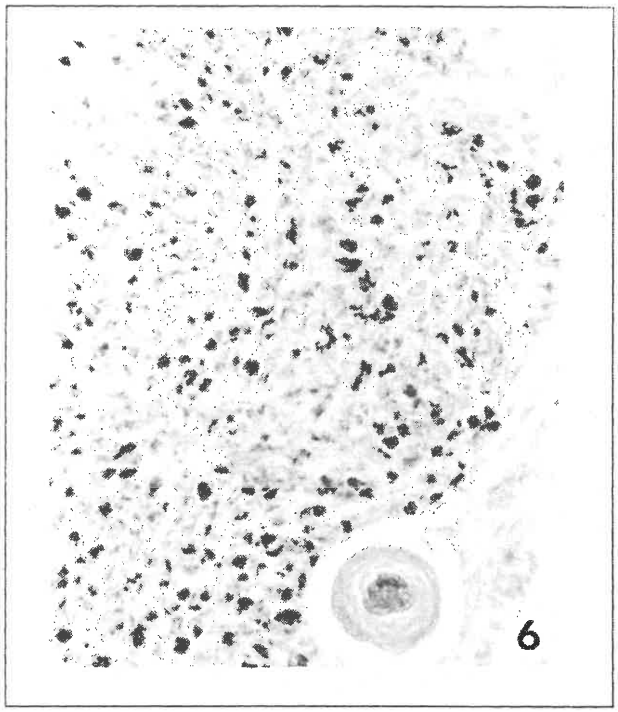

Figure 6. Acid-fast structures (dark deposits), possibly bacteria, in a solid inflammatory lesion in the peritoneal cavity of a control guppy, 24 week old. Ziel-Neelsen stain.

Regression Analysis of DMBA Study

Lesion morphology, age, presence of AFB, and sex were entered into the regression model for the DMBA study (Table 3). Data for medaka and guppies were analyzed as one pooled group. The severity rating correlated positively with age, the presence of nonspecific inflammatory reactions, and the presence of AFB in the lesions. Also, males had significantly lower severity ratings.

Table 3
Stepwise regression analysis to examine factors most influencing the severity ratings of chronic inflammatory lesions in medaka and guppy exposed to DMBA

Model Statement: SR= SP SEX1 SEX2 AGE X1 X2 X3 X4 MORPH AFB

	Parameter				
	df	**SS**	**MS**	**F**	**P**
Regression	4	42.97	10.74	19.09	0.0001
Error	78	43.89	0.56		
Total	82	86.87			
	Beta	**SE**	**SS**	**F**	**P**
Intercept	2.12				
MORPH	-1.00	0.19	15.19	26.99	0.0001
AGE	0.03	0.01	10.05	17.86	0.0001
AFB	0.81	0.22	7.89	14.03	0.0003
SEX1	-0.42	0.18	3.12	5.55	0.0210
R^2= 0.4947					

Abbreviations: SR= severity rating; SP= species; SEX1= males; SEX2=females; X1= solvent control; X2= low dose; X3= intermediate dose; X4= high dose; MORPH= lesion morphology; AFB= acid fast bacteria; df = degrees of freedom; SS = Type II sum of squares; MS = mean square; F = F statistic; P = probability; Beta = partial regression coefficient estimating β (beta); SE = standard error.

Regression Analysis for AAF Study

Lesion morphology, species, age, and four exposure variables (AAF 6 hr x1, 12 hr x2, 12 hr x3, 12 hr x4) were entered into the regression model for the AAF study (Data for medaka and guppy were analysed as one pooled group, Table 4). As in the DMBA study, severity was positively related to age and the presence of nonspecific inflammatory reactions. Guppies had significantly higher severity ratings than the medaka. The severity rating was positively related to the 6 hr x 1 exposure variable and negatively related to the 12 hr x 2, 12 hr x 3, and 12 hr x 4 exposure variables.

With the exception of the AAF 12 hr x 2 exposure variable, all variables included in the regression models were significant at the 0.05 level. The model for the DMBA study explained 49% of the variance, whereas the AAF model explained only 12% of the variance in the data set.

Table 4
Stepwise regression analysis to examine factors most influencing the severity ratings of chronic inflammatory lesions in medaka and guppy exposed to AAF.

Model Statement: SR= SP SEX1 SEX2 AGE X1 X2 X3 X4 X5 X6 X7 MORPH					
Parameter					
	df	SS	MS	F	P
Regression	7	48.84	6.98	8.21	0.0001
Error	422	358.41	0.85		
Total	429	407.25			
	Beta	SE	SS	F	P
Intercept	2.44				
MORPH	-0.74	0.13	25.26	30.45	0.0001
X5	-0.53	0.15	11.02	12.97	0.0004
X2	0.41	0.12	9.19	10.83	0.0011
SP	-0.44	0.14	8.58	10.10	0.0016
AGE	0.01	0.00	5.55	6.53	0.0109
X6	-0.35	0.17	3.63	4.28	0.0392
X4	-0.23	0.15	1.97	2.32	0.1283
R^2= 0.1199	Abbreviations: SR= severity rating; SP= species; SEX1= males; SEX2=females; X1= constant control; X2= 6hr x 1; X3= 12hr x 1; X4= 12hr x 2; X5= 12hr x 3; X6= 12hr x 4; X7= constant AAF; MORPH= lesion morphology				

DISCUSSION

For medaka and guppies in the DMBA study, the prevalence of chronic inflammatory lesions decreased with increasing DMBA concentration, possibly as an indirect result of DMBA toxicity. In many cases, moribund specimens in the high DMBA exposure groups were sacrificed prior to the scheduled sampling dates. Possibly this did not allow time for development of the inflammatory lesions. Supporting this view is the fact that mycobacteria have a relatively long generation time. Alternatively, the sparsity of inflammatory lesions in the guppy and their absence in medaka from the high dose DMBA exposure may have resulted from the chemical suppression of the inflammatory response. DMBA is known to possess immunotoxic properties (Dean and Murray, 1991; Ward *et al.*, 1985). Furthermore, chi-square analyses clearly indicated a significantly lower prevalence of inflammatory lesions in those groups exposed to DMF. Perhaps the DMBA and DMF acted together to repress activation of inflammatory cells. Another possibility is that the DMBA or DMF exerted anti-microbial effects inhibiting proliferation of mycobacteria in the hosts and, consequently, the associated inflammatory lesions were diminished.

In the AAF study, the prevalence of inflammatory lesions appeared to fluctuate randomLy among test groups with no apparent trends. However, chi-square analysis of the medaka AAF test showed a significantly lower prevalence in pooled treatment groups compared to pooled controls. Similarly, this finding suggests an inhibitory effect on the inflammatory response associated with AAF exposure.

Medaka and guppy exhibited distinctly different morphological inflammatory responses. Lesions in the medaka were primarily diffuse reactions in the kidney, often with giant cells, that were not associated with the presence of acid-fast bacteria. Based on reports that giant cells are not typically associated with mycobacteriosis in fish, the absence of intact acid-fast bacteria, and the lesion morphology being inconsistent with descriptions of mycobacterial lesions in the Lature (Wolke and Stroud, 1978), a noninfectious or infectious agent(s) other than mycobacteria could have been responsible for the chronic inflammatory reaction in the medaka.

The guppies typically exhibited discrete nodular lesions, frequently in the liver, and AFB were repeatedly detected. Based on demonstration of AFB in lesions as sufficient evidence for diagnosis as mycobacteriosis (Snieszko, 1978), and the lesion morphology being consistent with descriptions by Wolke and Stroud (1978), 40% of the cases in the guppy in the DMBA study can be attributed directly to a naturally occurring mycobacterial infection.

The regression models revealed significant differences in the severity rating with respect to age, lesion morphology, AFB, species, sex, and some of the AAF exposure variables. Severity ratings were consistently higher in older fish and in cases exhibiting nonspecific inflammatory reactions. Conceivably, the older the specimen, the more likely it is to have encountered granuloma-inducing agent(s) and the longer the proliferative, chronic inflammatory reaction has had to expand, spread, and displace more normal host tissue. The positive correlation of the severity rating with nonspecific inflammatory reactions is also easy to conceptualize, in that the lesion is not confined to a nodular focus. Its relatively diffuse nature would explain the associated higher severity ratings as more normal tissue is replaced. Similarly, with the presence of acid-fast bacteria there is the potential for spread of the organisms, resulting in widely disseminated chronic inflammatory lesions in an effort to contain the mycobacterial agent.

Guppies not only were more severely affected by the lesions than were medaka in the AAF study, but guppies also exhibited an equal or higher incidence of chronic inflammatory lesions than medaka in all test groups in the DMBA and AAF studies. Perhaps the medaka is less susceptible to the agents, including mycobacteria, responsible for the inflammatory lesions.

The reason for the lower severity ratings in male guppies from the DMBA study may be attributable to a venereal mode of transmission which predisposes the females to infection. At the time of fertilization, the gonopodium of the male enters the vent of the female delivering sperm which fertilizes the eggs. According to Anderson (1990), infectious agents in fish can be spread through the transfer of contaminated sex products. This is not an issue in the medaka whose eggs are externally fertilized. Although there was no evidence of chronic inflammatory lesions in developing guppy embryos, extensive granulomatous lesions were frequently present in the peritoneal cavity along the ovarian lining. If the infectious agent was present in the ambient water, contact between male and female guppies during sperm transfer could have resulted in the development of lesions in the peritoneal cavity in females, which would explain the lower severity ratings in males of the DMBA study.

In many of the AAF-exposed guppies, there were slight cases of exclusively solid, nodular macrophage aggregates that were considered to be early stage granulomatous lesions. Possibly some of these cases were actually melanomacrophage centers with little pigment deposition that were mistaken for early granulomatous lesions. Because these cases were typically assigned a rating of 1, an explanation is provided for the entry of the AAF exposure variables into the regression

model and their negative correlation with the severity rating. It is not surprising that the early, solid granulomatous lesions could be confused with melanomacrophage centers because of the ambiguous staining characteristics of hematoxylin and eosin. Melanomacrophage centers and granulomatous lesions are morphologically and functionally similar, as they are both discrete aggregates of macrophages that have a defensive role in sequestering antigens. Antigens and other particulates are deposited within melanomacrophage centers and, consequently, melanomacrophage centers may become foci for granuloma formation, especially in diseases such as mycobacteriosis where the antigen persists (Ferguson, 1989).

Exposure to DMBA and AAF was not responsible for the formation of inflammatory lesions based on the finding that there was an equally high or higher prevalence of inflammatory lesions in pooled controls when compared to pooled treatment groups in both studies. There was, however, evidence that all three chemical compounds (DMBA, AAF, and DMF) involved in this study exerted suppressive effects on the inflammatory responses of test species.

Inflammatory lesions in guppies were at least in part associated with acid-fast bacteria, presumed to be mycobacteria. Macrophages, which are traditionally believed to be the primary constituent of inflammatory lesions, play an important role in tumor surveillance and, according to Adams (1976), mycobacteria stimulate macrophages in such a way that neoplasia is inhibited and may even be reversed. Thus, an explanation is possibly provided for the fact that there were few cases of concomitant inflammatory lesions and neoplasms in this study. Perhaps those test specimens with inflammatory lesions possessed macrophages that were better able to ward off neoplastic cells.

In conclusion, the impact of a naturally-occurring mycobacterial infection on the immune system and the resultant influence on induction or suppression of chemically-induced neoplasia are not clear. In any case, attempts should be made to eliminate this infectious agent from test specimens that are to be used in carcinogenesis bioassays.

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