Reprinted from

MODELS FOR ENVIRONMENTAL TOXICOLOGY/BIOMARKERS, IMMUNOSTIMULATORS

Volume 1
1994

Development and Use of Medaka as a Model for Immunotoxicity

Lorraine E. Twerdok, E. Maxine Boncavage-Hennessey, Robert A. Finch and Henry S. Gardner

SOS PUBLICATIONS FAIR HAVEN, NJ, USA

Chapter 14

Development and Use of Medaka as a Model for Immunotoxicity

Lorraine E. Twerdok[1], E. Maxine Boncavage-Hennessey[1], Robert A. Finch[2] and Henry S. Gardner[2]

[1]GEO-CENTERS, INC.
Frederick, MD USA

[2]U.S. Army Biomedical Research and Development Laboratory
Fort Detrick
Frederick, MD, USA.

ABSTRACT

Several species of teleosts, including medaka (*Oryzias latipes*), have been studied with respect to neoplastic responses to known carcinogens. Little information, however, is available concerning the responses of the immune system to environmental chemicals in the medaka. We have initiated studies to characterize immune organs and function in medaka, with the goal of using the medaka as a predictive model of immunotoxicity in vertebrates. Development of a teleost model for immunotoxicity is part of an integrated biological approach for assessment of the hazard resulting from exposure to complex chemical contaminants in the environment. Medaka immune organs essential for leukopoiesis/erythropoiesis as well as cell morphology were examined. Cells isolated from the anterior kidney, spleen and whole blood were characterized with respect to nonspecific esterase activity, myeloperoxidase activity and acid phosphatase activity. *in vitro* phagocytic cell function following *in vitro* activation was assessed in primary cultures of pronephros adherent cells. Data collected to date characterizing the endogenous bacterial flora of medaka cultured in our laboratory is also presented.

INTRODUCTION

It has become increasingly evident over the last decade that the immune system is an important target organ for toxicity (U.S.Congress, 1991; Vos *et al.*, 1989; Dean *et al.*, 1982). Although immunotoxicology as a discrete field of study is advancing rapidly, the systematic study of immunotoxicity is complicated by the integrated nature of the immune system and the paucity of human exposure data available. Furthermore, there is currently no fully validated "battery" of short-term screening assays available to detect and assess potential immunologic toxicants (U.S.Congress, 1991; Luster *et al.*, 1992). A variety of test systems to assess immunocompetence is necessary due to the diverse and often overlapping mechanisms of defense utilized by the immune systems in response to immunologic challenge. Immunotoxicity can be manifested when one or more system(s) is compromised. Conversely, suppression or hyperactivation of one immune

function may have no adverse effect on overall host immunocompetence. Therefore, demonstration of *in vivo* adverse effects is critical in applied immunotoxicology research.

The overall goal of the research performed in our laboratory is to develop an integrated biological approach to assessment of the hazard resulting from exposure to complex chemical contaminants in the environment. Existing components of this program for hazard assessment in the field include acute toxicity testing by ventilatory monitoring (Feder *et al.* 1992), teratogenicity assessment using frog embryos (Bantle *et al.*, 1991; American Society for Testing and Materials, 1991), carcinogenicity/tumor promotion assessment using medaka (Hawkins *et al.*, 1985, 1985; Battalora *et al.*, 1990), as well as Daphnia toxicity assays (Janssen and Persoone, 1993), and genetic toxicity assays using *in vitro* assays such as the Ames test (Ames *et al.*, 1975; Maron and Ames, 1983) and the CHO sister chromatid exchange assay (Wolff and Perry, 1974; Latt *et al.*, 1981). Due to recent advances in immunological research which demonstrate the importance of the immune system in many disease processes and chemically-induced toxicities, it is necessary to include detection of immunotoxicity into any integrated biological approach to hazard assessment. Thus, the specific aim of this research project is to develop and validate immunotoxicologic screening assays employing teleosts as the model system. The work presented here discusses our initial studies to characterize specific parameters of the immune system of the medaka (*Oryzias latipes*).

MATERIALS AND METHODS

Test species

Japanese medaka (*Oryzias latipes*) were reared in the laboratory in a flow-through well-water system maintained at 25°C with 16/8 hr light/dark cycle, and fed flake food (Tetramin®), brine shrimp and microworms. Five to seven month old medaka (500-600mg) which had received no treatment or stressful handling were used in all studies unless otherwise noted. Only fish which appeared healthy and active were used in these tests and groups were selected by age, without regard to sex.

Chemicals and Materials

L15 media, fetal bovine serum (FBS), glutamine and antibiotics were obtained from GIBCO (Grand Island, NY). Ninety-six well microtiter plates were obtained from Nunc, INC. (Naperville, IL). Luminol (3-aminophthalhydrazide) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were obtained from Sigma Chemical Co., (St. Louis, MO).

Anterior kidney and spleen cell isolation

Fish were anesthetized with 200 mg/L MS-222 (tricaine methanesulfonate) and sacrificed by cervical transection. Anterior kidney and/or spleen were collected and pooled in 2-3 ml L15 media or fish physiological saline (FPS), then homogenized in glass/glass homogenizers.[Fish physiological saline consists of 110 mM NaCl (6.44 mg/L), 0.15 mM KCl (11 mg/L), 0.2 mM $CaCl_2$ (22 mg/L), $MgSO_4$ (12 mg/L), KH_2PO_4 (7 mg/L) and $NaHCO_3$ (10 mg/L) in distilled H_2O.] Cell suspensions were passed through glass wool to remove cellular debris and red blood cells and centrifuged at 350 xg for 15 min, washed twice with FPS, resuspended in 0.5 ml of FPS and counted using a hemocytometer. Cells from these whole organ homogenates were plated into 96-well microtiter plates at 2×10^5 cells per well and either used immediately (for special staining) or after 48 hr culture at 30°C in L15 media supplemented with 5% FBS, 2 mM glutamine, 100 IU/ml

penicillin and 100 µg/mL streptomycin. Cultured cells were washed after 24 hr to remove unattached cells and debris.

In later experiments, cells isolated from the anterior kidney were further purified by density gradient separation (Secombes, 1990). Three mL of the whole organ cell homogenate was layered onto 10 mL of a 51% Percoll® solution brought to isotonicity using 10X Hank's Balanced Salt Solution (HBSS), centrifuged for 30 min at 400 xg, and the fraction containing monocytes (a whitish band below the darker band of cellular debris at approximately the 50-60% fractions) collected. The monocyte-containing fraction was diluted with 5-10 mL FPS, washed twice and counted in a hemocytometer. Density gradient-enriched anterior kidney cells were then plated into 96-well microtiter plates at 2 x 10^6 cells per well and used immediately or incubated for attachment for 90 min at 25°C in L15 supplemented with 2% stock medaka serum (prepared as described below). After attachment, cells were either used immediately or cultured for 1.5, 24, 48 or 72 hr at 25°C in L15 supplemented with 5% FBS, glutamine and antibiotics. Cultured cells were washed twice at 1.5 and 24 hr to remove unattached cells and debris.

Blood cell and serum isolation

Anesthetized (MS-222, 200 mg/L) fish were cut into thirds and bled into 5 mL FPS + EDTA. Cells were centrifuged, supernatant collected, cell pellet resuspended in a small amount of FPS and the cells counted by hemocytometer for use in staining experiments. The supernatant containing fish serum was then further diluted with FPS to make a "stock" serum solution of 20 mL FPS (containing serum from 20 medaka). Assuming a recovery of approximately 10 µl serum per fish, the concentration of the stock serum solution was ≈ 1% serum in FPS (v/v). The solution was then filtered through a 0.22 µ filter and frozen at -20°C until needed for assays. Prior to use, stock serum was heated in a waterbath at 56°C for 20 min (DeKoning and Kaattari, 1992) to inactivate the complement.

Cell staining

Esterase, acid phosphatase, and myeloperoxidase (MPO) enzymatic activities associated with cells from the anterior kidney, spleen and blood were assessed by special staining techniques. Slides were prepared using ≅ 10^5 cells from each specified organ preparation using a cytocentrifuge. Slides were then stained for esterase activity [using two substrates,α-naphthyl acetate and/or naphthol AS-D chloroacetate (Sigma cat. # CP-1, Procedure No. 91)], acid phosphatase activity (Sigma cat. # CP-1, Procedure No. 181) or MPO activity (Sigma cat. # CP-1, Procedure No. 390).

Superoxide anion production

Superoxide dismutase-inhibitable extracellular and intracellular superoxide anion production was detected by assessing reduction of cytochrome *c* or nitroblue tetrazolium (NBT), respectively (Secombes *et al.* 1988; Jensen *et al.* 1991; Zelikoff and Enane, 1991; Secombes, 1990). Whole organ anterior kidney or spleen cell preparations were plated at 2 x 10^5 cells per well and assayed for superoxide anion (O_2^-) production 48 hr later. Attached cells were stimulated with 1 µg/mL 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and the amount of superoxide dismutase-inhibitable O_2^- produced was assessed 15 min after stimulation by spectrophotometric measurement in a microtiter plate reader at 550nm (Abs_{550}) or 620nm (Abs_{620}) for cytochrome *c* or NBT, respectively. The changes in absorbance were then used to calculate the amount of cytochrome *c* or NBT reduced

according to the method of Pick (Pick and Mizel, 1981; Pick *et al.* 1981). Results are presented as nmol cytochrome *c* or NBT reduced per 2×10^5 cells. Results are adjusted for nonspecific reduction of ferricytochrome *c* by subtraction of non-superoxide dismutase-inhibitable production. Cell preparations enriched for monocytes/macrophages by Percoll® separation and attachment to plastic were assayed for superoxide anion production by cytochrome *c* reduction after 0, 1.5, 24, 48 and 72 hr in culture. Cells were washed twice with HBSS, incubated with cytochrome *c* (C_f=1.6 mg/mL) with and without superoxide dismutase (SOD; C_f=50 µg/mL), and then stimulated with TPA (C_f=1 µg/mL) or solvent controls. Abs_{550} was measured every 15 min for three hr and results calculated as described above.

Hydrogen peroxide production

Hydrogen peroxide (H_2O_2) production following TPA-stimulation was assessed using peroxidase-dependent oxidation of phenol red (Secombes, 1990). Assay conditions were the same as those described for O_2^- production for whole organ preparations. For these studies, H_2O_2 production was not assessed in monocyte/macrophage enriched preparations.

Measurement of chemiluminescence (CL) responses. CL was monitored in freshly isolated anterior kidney cells in an ambient temperature liquid scintillation counter (Packard 2500TR) in the Single Photon Mode. Samples contained 2×10^6 anterior kidney cells and luminol (C_f=25 µM) in a total volume of 2 mL. Reactions were initiated by the addition of TPA (C_f=1 µg/mL) to the samples and monitored for 2 hr. Results are expressed as counts per unit time minus background. Data are expressed as peak cpm (maximum responses) and area under the curve (AUC; counts x min). Area under the curve was approximated using the trapezoid method.

Bacterial isolation and identification. Characterization of bacterial flora of presumed enteric, skin (mucus) and gill origin was accomplished by isolating bacteria from the water of aquaria containing 6 month old fish. Fish which appeared healthy and active were transferred into clean aquaria and held, without feeding, under static conditions. Seventy-two hr after the addition of fish, water samples were taken for bacterial isolation and identification. Approximately 500 mL aquarium water was filtered through a 0.22 µ filter and the filter was inverted and "streaked" onto tryptic soy agar (TSA). The filter was then removed and discarded and the plates were incubated at 25°C. After 48 hr, colonies of different macroscopic appearance were restreaked onto fresh TSA and incubated at 25°C. This process was repeated until pure cultures were obtained. These pure cultures were then sent out to commercial laboratories for identification by morphological and biochemical methods. Characterization of endogenous bacterial flora in intact fish was determined in 15 month-old medaka which had been used as a breeding population between 5 and 9 months of age. These animals did not demonstrate any external symptoms of disease, but had undergone significant handling- and age-related stress. Ten fish were euthanized with MS-222 (400 mg/L), necropsied aseptically and tissue samples from various organs including spleen, anterior kidney, liver, heart, lining of the peritoneal cavity or any macroscopic lesion (*e.g.* eye) were streaked onto TSA. Pure cultures of bacteria were isolated and identified as described above.

RESULTS AND DISCUSSION

The data presented in this paper are from initial experiments designed to develop a teleost model for immunotoxicity. This information will then be incorporated into an integrated biological

approach for assessment of the hazard resulting from exposure to complex chemical contaminants in the environment. The initial steps in developing a teleost model for use in immunotoxicity screening assays is selection of species and characterization of immune organs from that species. We have selected the medaka, *Oryzias latipes*, for use as a teleost model of immunotoxicity for four reasons; (1) ease of culture and hardiness of medaka in general (Kirchen and West, 1969), (2) the genetics of medaka are well-characterized (Ijiri, 1987; Naruse *et al.*, 1988), (3) the susceptibility of medaka to chemical carcinogens is well-characterized and appears mechanistically analogous to mammalian chemically-induced carcinogenic processes (Gardner *et al.*, 1990; Van Beneden *et al.*, 1990; Hyodo-Taguchi *et al.*, 1985, 1989; Hawkins *et al.*, 1985,1988), (4) the small size of the fish will permit the use of large numbers of animals for laboratory or field exposure. It is anticipated that a large sample size will be necessary to detect toxicity at the concentrations of chemical contaminants found in the environment.

Characterization of the immune organs of medaka included cell yields and viability, staining characteristics for non-specific esterase, acid phosphatase and MPO activities, phagocytic cell function, and preliminary characterization of endogenous bacterial flora. Cell yields and viability as measured by trypan blue exclusion are given in Table 1. Enrichment of anterior kidneys for macrophages/monocytes decreased yields by 50-60% as compared to the whole organ cell preparation yields. Cell viability as measured by trypan blue exclusion was routinely ≥ 95% for anterior kidney preparations and ≥ 90% for spleen cells. Cell yields were not calculated for blood, *i.e.* cells collected from fish bled into FPS.

Table 1.
Average cell yields from anterior kidney and spleen of Japanese medaka

	Cell Yield* (# cells x 10^6/animal)	Viability (% cells excluding trypan blue)
Anterior kidney		
Whole organ	1.99 ± 0.46	95.0 ± 1.24
Enriched preparation	0.88 ± 0.01	97.9 ± 0.45
Spleen		
Whole organ	1.85 ± 0.46	90.0 ± 2.59

*Values represent Mean ± SEM, n ≥ 3.

Whole organ cell population differentials for non-specific esterase, acid phosphatase and MPO activities are given in Figures 1 and 2. As expected, many cells of head kidney and spleen origin were positive for esterase and acid phosphatase activities, indicating the presence of monocytic cells and those of lymphocyte origin, respectively. The percent of cells stained positively for esterase activity assessed using the double-staining technique (two substrates as opposed to a single substrate) was 59.4 ± 4.72 (values given are Mean ± SEM) and 50.9 ± 12.8 in anterior kidney and spleen cells, respectively; 14 .4 ± 2.81% and 8.02 ± 1.70 % of cells stained positively for acid phosphatase activity, respectively. While present, the percent of esterase and acid phosphatase positive cells was much lower in cells found in the blood. Interestingly, a considerable number of cells were MPO positive, 24.7 ± 1.27 %, 20.8 ± 16.2 % and 3.50 ± 2.29 % from anterior kidney, spleen and blood, respectively, presumably indicating a significant number of neutrophils in the

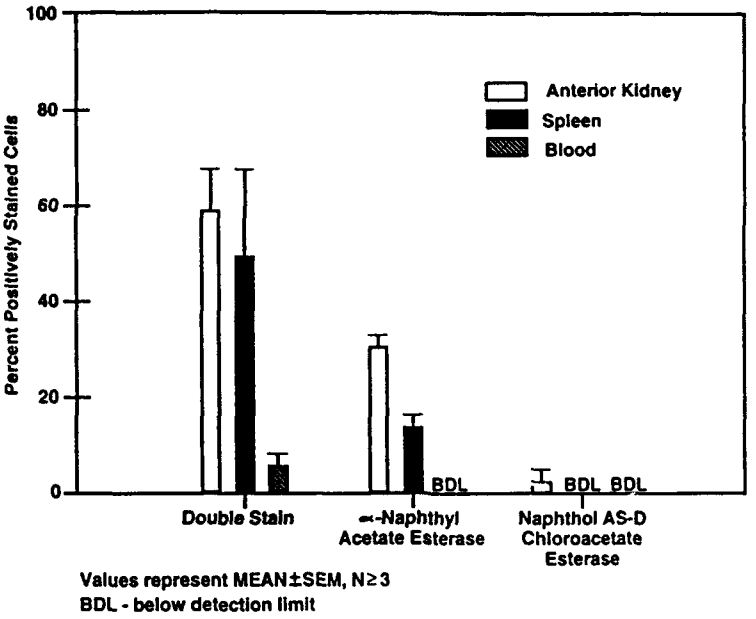

Figure 1. Esterase staining characteristics in medaka whole organ preparations.

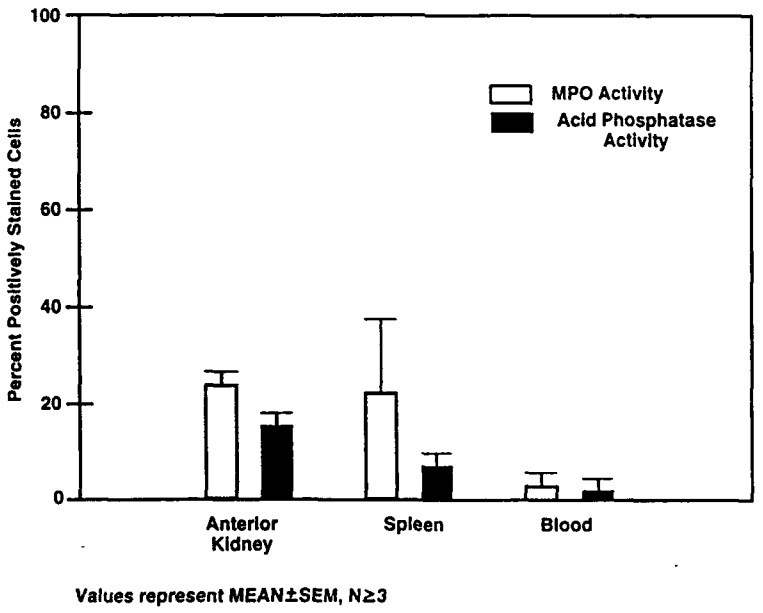

Figure 2. MPO and acid phosphatase staining characteristics in medaka whole organ preparations.

medaka. We plan to investigate these findings further with assessment of enzymatic activity in cytosol preparations. It should be noted that a large amount of variability in staining differentials was observed in the spleen as compared to the anterior kidney.

Measurement of reactive oxidant generation was assessed *in vitro* by measurement of reactive oxidants following stimulation by phorbol ester. Initial experiments demonstrated measurable

Table 2
Oxidant generation by TPA-stimulated anterior kidney and spleen cells of japanese medaka[a]

	ANTERIOR KIDNEY[b]	SPLEENb
Superoxide anion production[c] (nmol reduced/2 x 10^5 cells plated)		
Cytochrome *C*	0.536 ±0.137	0.03 ±0.001
Nitroblue tetrazolium	0.513 ± 0.013	BDL[d]
H_2O_2 production (nmol reduced/2 x 10^5 cells plated)		
Phenol red	0.426 ± 0.030	0.090 0 ± 008

production of superoxide anion and hydrogen peroxide by activated anterior kidney or spleen cells from whole organs (Table 2). Luminol-amplified oxidant-dependent generation of chemiluminescence was demonstrated in TPA-stimulated anterior kidney whole organ preparations (Table 3).

Table 3.
Quantitation of the luminol-amplified chemiluminescent response from TPA-stimulated anterior kidney cells

SOURCE OF CELLS	AUC[a] CPM [(cpm x min) x 10^8]	PEAK (cpm x 10^6)
Whole Organ Preparation	3.22 ± 0.58[b]	6.03 ± 1.33

[a]AUC = area under the curve as estimated by the trapezoid method.
[b]values represent MEAN ± 5 , SEM, n ≥ 5.

In an effort to increase the size and reproducibility of the oxidant response in anterior kidney-derived cells, the whole organ preparations were enriched for monocyte/macrophage content by density gradient separation followed by attachment to plastic. Production of superoxide anion after stimulation was then assessed immediately after Percoll® separation and 1.5, 24, 48 and 72 hr after attachment to tissue culture vessels. It was hypothesized that activity would increase with culture time as monocytes differentiated into macrophages and the data presented in Figure 3 supported this hypothesis. There was no detectable production of superoxide anion within 180 min after addition of TPA at 0 hr post-attachment (immediately after Percoll® separation) or 1.5 hr post-attachment; production peaked at 24 hr post-attachment and returned to baseline by 72 hr post-attachment (Figure 3). Data from 72 hr post-attachment assays are not given. At 24 hr and 48 hr

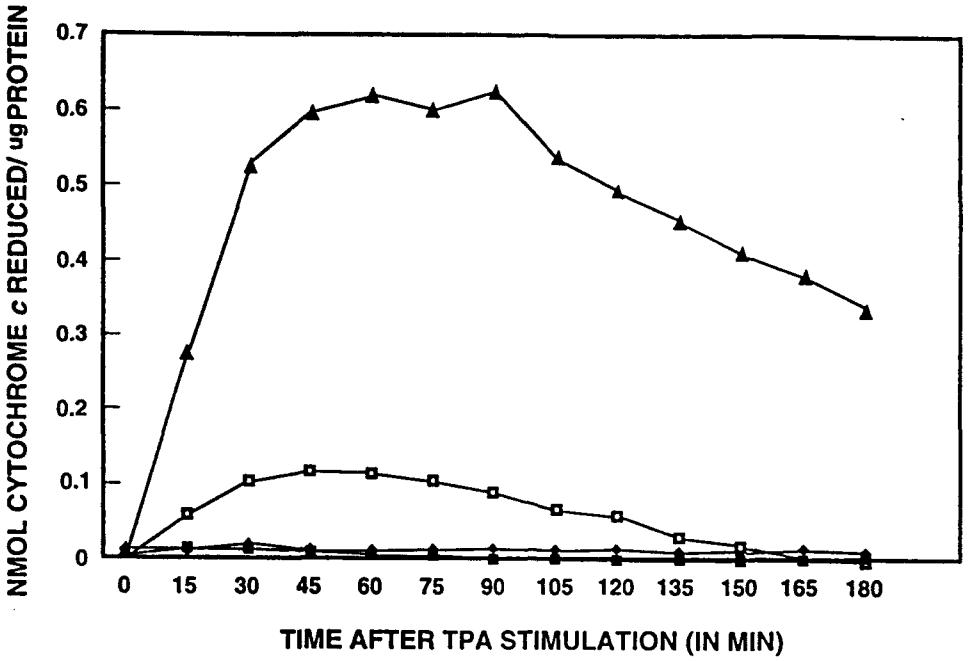

Figure 3. TPA-stimulated SOD-inhibitable superoxide anion generation in macrophage-enriched anterior kidney preparations was monitored for 180 min at various times after cellular attachment to plastic in culture: 0 hr post-attachment (no attachment) ■, 1.5 hr post-attachment ◆, 24 hr ▲, 48 hr post-attachment □, (72 hr post-attachment data not shown).

post-attachment, production of superoxide anion in monocyte/macrophage enriched cells peaked 45 min to 1 hr after stimulation with TPA. These data suggest that a significant number of "resting" or "resident" monocytes/macrophages reside in the anterior kidney of medaka which can be matured via attachment to plastic to a mature state responsive to TPA stimulation as demonstrated by a subsequent respiratory burst. Based on change in absorbance data, the values observed for superoxide anion production by medaka anterior kidney cells are comparable to those obtained for rainbow trout by other investigators (Secombes *et al.*, 1988; Secombes and Chung, 1988). However, recent data generated in our laboratory (data not shown) suggest that stimulated trout macrophages generate greater absolute quantities of superoxide anion than medaka-derived macrophages. Further characterization and optimization of oxidant generation in medaka cells is in progress.

The final parameter characterized was identification of endogenous bacterial flora. Since future experiments will incorporate an *in vivo* pathogenic challenge or *in vivo* immunogenic challenge for primary antibody response, it is necessary to characterize the composition of the normal bacterial flora in the test species. It should be noted, however, that the composition of bacterial flora can change significantly with water and food sources, and the results presented here are most likely location- and culture-specific. No bacteria were isolated on tryptic soy agar following repeated culturing of processed water before entry into culture tanks. *Acinetobacter, Pseudomonas, Enterobacter* and *Klebsiella* were isolated from the culture water of 6 month old fish (Table 4). Water borne bacteria were assumed to be primarily of enteric, scale/mucus and gill origin, since no organisms were isolated from culture water before the introduction of fish. Bacteria isolated from the internal organs of 15 month old medaka included *Aeromonas, Pleisonmonas* and *Pasteurella* species (Table 5). These data represent preliminary studies, and a more comprehensive characterization of the endogenous flora of medaka from our facility, as well as from other culture facilities, is planned.

Table 4.
Bacterial flora of medaka exterior surface and enteric origin[a]

Acinetobacter calcoaceticus (var. *anitratus*)
Acinetobacter calcoaceticus (var. *lwoffi*)
Pseudomonas species (morphology one)
Pseudomonas species (morphology two)
Aeromonas caviae Enterobacter cloacae
Klebsiella pneumoniae
Pseudomonas species

[a]Bacteria were isolated on tryptic soy agar from water from aquaria containing 6 month old fish held, without food, for 72 hr under static conditions.

Table 5
Results of bacterial cultures from internal organs of 15 month old medaka

Sample Site	Organism(s) Isolated	Antibiotic Sensitivity	
		Resistant	Sensitive
Posterior abdominal wall	*Aeromonas hydrophilia*	ampicillin	trimethoprim/ sulfamethoxazole
		tetracycline	cefamandole
		chloramphenicol	cifonitin
			ciprofloxacin
			gentamycin
			tobramycin
			ticarcillin
	Acinetobacter lwoffi	none	all of above
	Pleisomonas species	none	all of above(chloramphenicol not run)
Eye	*Pasteurella species*	tetracycline chloramphenicol	all of above
Anterior kidney	*Pasteurella species*	tetracycline	all of above
Spleen	*Pasteurella species*	tetracycline	all of above

Note: anterior kidney and spleen samples were from the same fish

The purpose of this work was to begin to characterize the medaka immune system with the goal of using this particular fish species for immunotoxicity hazard assessment of contaminants in the environment. *In vitro* generation of reactive oxidants by anterior kidney monocytes/macrophages following phorbol ester stimulation was analogous to similar experiments in rainbow trout, suggesting that the medaka may provide a relevant model for assessment of immunotoxic hazard.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the excellent technical support of Ronald Miller, Jr., Margaret W. Toussaint and Michael Westberg in the rearing, care and maintenance of the fish used in these studies.

REFERENCES

American Society for Testing and Materials (1991). Standard guide for conducting the frog embryo teratogenesis assay-*xenopus* (Fetax). ASTM 1439-91. ASTM, Philadelphia, PA.

Ames, B.N., McCann, J. and E.Yamasaki (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res. 31:347-364.

Bantle, J.A., Dumont, J.N., Finch ,R.A. and G. Linder (1991). Atlas of Abnormalities: A Guide for the Performance of FETAX. Oklahoma State Publications Department, Stillwater, OK.

Battalora, M.St.J., Hawkins, W.E., Walker, W.W. and R.M .Overstreet (1990). Occurrence of thymic lymphoma in carcinogenesis bioassay specimens of the Japanese medaka (*Oryzias latipes*). Cancer Res. 50:5675s-5678s.

Dean, J.H., Luster, M.I. and G.A. Boorman (1982). Methods and approaches for assessing immunotoxicity: An overview. Environ. Hlth. Perspect. 43:27-29.

DeKoning, J. and S.L. Kaattari (1992). Use of homologous salmonid plasma for improved responsiveness of salmonid leukocyte cultures. *In:* Techniques in Fish Immunology-2.(J.S. Stolen, T.C. Fletcher, D.P. Anderson, S.L. Kaattari and A.F. Rowley, editors). SOS Publications, Fair Haven, NJ. pp. 61-65.

Feder, P.I., Lordo, R.A., Shedd, T.R., van der Shalie, W.H., Leach, J.D. and H.S. Gardner (1992). On-line statistical analysis of fish ventilatory response data. Society of Environmental Toxicology and Chemistry 13th Annual Meeting Abstracts, pp. 247.

Gardner, H.S., van der Schalie, W.H., Wolfe, M.J. and R.A. Finch (1990). New methods for on-site biological monitoring of effluent water quality. *In*: *In situ* Evaluations of Biological Hazards of Environmental Pollutants.(S.S. Sandhu, editor). Plenum Press, New York, pp. 61-69.

Hawkins, W.E., Overstreet, R.M., Fournie, J.W. and W.W. Walker (1985). Development of aquarium fish models for environmental carcinogenesis: Tumor induction in seven species. J. Appl. Toxicol. 5:261-264.

Hawkins, W.E., Overstreet, R.M. and W.W. Walker (1988). Carcinogenicity tests with small fish species. Aquatic Toxicol. 11:113-128.

Hyodo-Taguchi, Y. and N. Egami (1985). Establishment of inbred strains of the medaka *Oryzias latipes* and the usefulness of the strains for biomedical research. Zool. Sci. 2:305-316.

Hyodo-Taguchi, Y. and N. Egami (1989). Use of Small Fish in Biomedical Research, with special reference to inbred strains of medaka. *In*: Nonmammalian Animal Models for Biomedical Research. (A.D. Woodhead, editor). CRC Press, Inc., Boca Raton, pp. 185-214.

Ijiri, K. (1987). A method for producing clones of the medaka, *Oryzias latipes* (Teleostei, Oryziatidai). *In*: Proceedings, V Congress of European Ichthyologist, Stockholm 1985. (S.O. Kullander and B. Fernholm, editors.) Dept. Vertebrate Zoology, Swedish Museum of Natural History, Stockholm, Sweden, pp. 277-283.

Janssen, C.R. and G. Persoone (1993). Rapid toxicity screening tests for aquatic biota. 1. Methodology and experiments with *Daphnia magna*. Environ. Toxicol. Chem. 12:711-717.

Jensen, E.G., Skaare, J.U., Egaas, E. and A.Goksoyr (1991). Response of xenobiotic metabolizing enzymes in rainbow trout (*Oncorhynchus mykiss*) to endosulfan, detected by enzyme activities and immunochemical methods. Aquat. Tox. 21:81-92.

Kirchen, R.A. and W.R. West (1969). The Japanese Medaka: Its Care and Development. Carolina Biological Supply Company, Burlington, NC.

Latt, S.A., Allen, J., Bloom, S.E., Carrano, A., Falke, E., Kram, D., Schneider, E., Tice, R., Witfield, B. and S. Wolff (1981). Sister-chromatid exchanges: a report of the Gene-Tox Program. Mutat. Res. 87:17-81

Luster, M.I., Portier, C., Pait, D.G., White,Jr., K.L., Gennings, C., Munson, A.E. and G.J. Rosenthal (1992). Risk Assessment in Immunotoxicology I. Sensitivity and predictability of immune tests. Fund. Appl. Tox. 18:200-210.

Maron, D.M. and B. Ames (1983). Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113:173-215.

Naruse, K., Shimada, A. and A. Ahima (1988). Gene-centromere mapping for 5 visible mutant loci in multiple recessive tester stock of the medaka (*Oryzias latipes*). Zoo. Sci. 5:489-492.

Pick, E., Charon, J. and D. Mizel (1981). A rapid densitometric microassay for nitroblue tetrazolium reduction and application of the microassay to macrophages. J. Reticuloendothel. Soc. 30:581-593.

Pick, E. and D. Mizel (1981). Rapid microassays for the measurements of superoxide anion and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Methods 46:211-226.

Secombes, C.J., Chung, S. and A.H. Jeffries (1988). Superoxide anion production by rainbow trout macrophages by the reduction of ferricytochrome c. Dev. Comp. Immunol. 12:201-206.

Secombes, C.J. and S. Chung (1988). Analysis of events occurring within teleost macrophages during the respiratory burst. Comp. Biochem. Physiol. 89B:539-544.

Secombes, C.J. (1990). Isolation of salmonid macrophages and analysis of their killing activity. *In*: Techniques in Fish Immunology. no. 1. (J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson and W.B. van Muiswinkel, editors). SOS Publications, Fair Haven, NJ, pp. 137-154.

U.S.Congress, Office of Technology Assessment (1991). Identifying and Controlling Immunotoxic Substances - Background Paper, OTA-BP-BA-75. U.S. Government Printing Office, Washington, D.C.

Van Beneden, R.J., Henderson, K.W., Blair, D.G., Papas, T.S. and H.S. Gardner (1990). Oncogenes in hematopoietic and hepatic fish neoplasms. Cancer Res., (SUPPL.) 50:5671s-5674s.

Vos, J., Van Loveren, H., Wester, P. and D. Vethaak (1989). Toxic effects of environmental chemicals on the immune system. Trends Pharamacol. Sci. 10:289-292.

Wolff, S. and P. Perry (1974). Differential Giemsa staining of sister chromatids and the study of sister chromatid exchange without autoradiography. Chrom. 48:341-353.

Zelikoff, J.T. and N.A. Enane (1991). Assays used to assess the activation state of rainbow trout peritoneal macrophages. *In:* Techniques in Fish Immunology-2. (J.S. Stolen, T.C. Fletcher, D.P. Anderson, S.L. Kaattari and A.F. Rowley, editors). SOS Publications, Fair Haven, NJ, pp. 107-124.

Disclaimer

The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as official Department of the Army position, policy or decision, unless so designated by other official documentation. Citations of commercial organizations or trade names in this manuscript do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the **Care and Use of Laboratory Animals,** NIH publication 86-23, 1985 edition.