

Effect of *in vivo* pentachlorophenol exposure on *Fundulus heteroclitus* phagocytes: modulation of bactericidal activity*

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ABSTRACT: The effect of pentachlorophenol (PCP) on reactive oxygen intermediate (ROI)-mediated bactericidal activity was studied in an estuarine teleost, *Fundulus heteroclitus*. Fish were exposed *in vivo* for 12 d to 50 $\mu\text{g l}^{-1}$ (nominal) PCP, an environmentally relevant, sublethal concentration. Fish were then sacrificed, and macrophages and eosinophils isolated from the pronephros. Cells were assayed for their ability to phagocytose FITC-conjugated yeast, and produce the ROIs superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Cells were also assayed for luminol-enhanced chemiluminescence (LCL), a measure of activity of the H_2O_2 -peroxidase-halide bactericidal system. Finally, cells were tested for bactericidal activity against *Listonella anguillarum*, a marine bacterium responsible for vibriosis in fish. In unexposed fish, eosinophils were more active than macrophages in defense related mechanisms. The O_2^- production, LCL activity, and bactericidal activity of phagocytosing eosinophils were significantly greater than macrophages; higher levels of phagocytosis and H_2O_2 production by eosinophils were also observed. Significant reductions in eosinophil phagocytic activity were produced by PCP exposure. Phagocytes from PCP-treated fish showed decreased bactericidal activity, which was statistically significant in the case of macrophages. Both phagocytic cell types had significantly increased basal levels of O_2^- and LCL after PCP treatment. However, PCP had no effect on the phagocytically stimulated levels of these activities, nor on the stimulated production of H_2O_2 . Modulation of immune activities of these phagocytic cells by xenobiotics, as seen in these experiments, has the potential to alter susceptibility to disease causing agents such as *Listonella*.

KEY WORDS: *Fundulus heteroclitus* · Immunotoxicity · Phagocytes · Bactericidal activity · Reactive oxygen intermediates

INTRODUCTION

Pentachlorophenol (PCP) and its sodium salt (NaPCP) are widely used pesticides, with total worldwide production estimated at 50 million kg yr^{-1} (Eisler 1989). Although PCP use in the United States is now limited to the wood and paper industry, its properties as a general biocide led to widespread use through the

1970s. In 1977, PCP was listed as the second most heavily used pesticide in the United States (Cirelli 1978). It is primarily through wood treatment plant effluents that PCP is introduced into the aquatic environment. Water concentrations of PCP associated with effluent spills range from 81 to 147 $\mu\text{g l}^{-1}$ (Pierce & Victor 1978). Others have reported water concentrations near wood treatment plants ranging from 0.005 to 40 $\mu\text{g l}^{-1}$; body burdens in fish taken from these areas were between 0.3 and 24 $\mu\text{g kg}^{-1}$ (Niimi & McFadden 1982, Niimi & Cho 1983).

Pentachlorophenol belongs to a class of chemicals whose toxicity results from their ability to uncouple oxidative phosphorylation (Weinbach & Garbus 1965). Exposure of fish to sublethal concentrations of PCP has been reported to result in behavioral and metabolic

*Preliminary data from this study were reported at the 8th International Symposium on Pollutant Responses in Marine Organisms

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changes. These include decreases in growth rate, food conversion efficiency, swimming performance, prey capture, and predator avoidance (Webb & Brett 1973, Mathers et al. 1985, Brown et al. 1987, Little et al. 1990, Samis et al. 1991). Chronic PCP exposures have also been shown to disrupt activities of citric acid cycle and phase I and II biotransformation enzymes, and to modulate hepatic and kidney glutathione levels (Boström & Johansson 1972, Arrhenius et al. 1977, Fox & Rao 1978, Meerman et al. 1980, Thomas & Wofford 1984).

In addition to these effects, PCP can act as an immunosuppressor in mammals and aquatic species. When hardshell clams *Mercenaria mercenaria* were exposed to sub-lethal concentrations of PCP, there was a dose-dependent decrease in clearance of bacteria from the hemolymph, as well as decreased cell-mediated bactericidal activity (Anderson et al. 1981, Anderson 1988). Previous work from this laboratory has shown that PCP can inhibit the formation of reactive oxygen intermediates (ROIs), a principle cell-mediated bactericidal mechanism. Inhibition of ROI production was seen in 2 species of fish, the Japanese medaka *Oryzias latipes* and the mummichog *Fundulus heteroclitus*. Following *in vitro* exposure of medaka phagocytes to PCP, there was a dose-dependent decrease in luminol-enhanced chemiluminescence (LCL) (Anderson & Brubacher 1993). Decreased LCL is indicative of reduced activity of the H₂O₂-peroxidase-halide antimicrobial system. When phagocytes from *Fundulus heteroclitus* were exposed *in vitro* to sub-lethal concentrations of PCP, decreased phagocytosis, O₂⁻ production, and bactericidal activity were seen (Roszell & Anderson 1994). However, 2 other aspects of ROI-mediated bactericidal activity, hydrogen peroxide (H₂O₂) production and LCL, were not affected (Roszell & Anderson 1993, 1996).

These previous *in vitro* screening studies suggested that PCP was a potential immunomodulator that might lower the resistance of fish to infection by pathogenic bacteria. Therefore, the purpose of the current work was to examine phagocytosis and ROI-mediated bactericidal activity of leukocytes following *in vivo* exposure of *Fundulus heteroclitus* to an environmentally relevant concentration of PCP.

MATERIALS AND METHODS

Fish. *Fundulus heteroclitus* were collected from a tributary of the Patuxent River (Maryland, USA) and transported immediately to Chesapeake Biological Laboratory. They were maintained in flow-through tanks for at least 2 mo prior to use.

Chemicals. Marine broth and agar used to grow *Listonella anguillarum* (ATCC strain 14181) were pur-

chased from Difco (Detroit, MI, USA). Pentachlorophenol (NaPCP) and pentafluorophenol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetic anhydride, benzene, and reagent grade hexane were obtained from J. T. Baker (Phillipsburg, NJ, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Pentachlorophenol exposure. Eight 10 l glass aquaria were supplied with filtered (5 µm) Patuxent River water (considered to be relatively pristine). Six fish (random sex, ~5 to 7 g) were placed in each tank, and allowed to acclimate for at least 2 wk. During this time the water was aerated, passed through activated charcoal filters, and held at 20 to 22°C.

A static-renewal system was used for PCP exposures. Fish were fed *ad libitum* each day 1 h before water changes; 50% of the water in all tanks was changed daily. Control tanks received filtered Patuxent River water; experimental tanks also received NaPCP to maintain a nominal concentration of 50 µg l⁻¹. All tanks were aerated to maintain oxygen saturation. Water temperature was held at 20 to 22°C; salinity varied with ambient conditions, and ranged from 13 to 16‰. Water conditions (pH, temperature, ammonia) were monitored daily.

Cell isolation. At the end of the exposure period, groups of fish (6 per group) were taken randomly from control or treated tanks. The pronephros (the primary source of phagocytic cells in fish) were removed and pooled in L-15 medium containing 1% antibiotic/antimycotic solution (10000 U ml⁻¹ penicillin G, 10 mg ml⁻¹ streptomycin, and 25 µg ml⁻¹ amphotericin B). To obtain a single cell suspension, the pronephros were disrupted with a Dounce tissue grinder. Enriched cultures of macrophages and eosinophils were then prepared using discontinuous Percoll gradients as previously described (Roszell & Anderson 1994). Cells were washed twice in L-15 medium supplemented with 5% fetal calf serum (FCS), and resuspended in L-15 medium supplemented with 5% FCS; final cell concentration was 2 × 10⁶ cells ml⁻¹.

Immunoassays. Phagocytosis, ROI production and bactericidal activity were measured as previously described (Roszell & Anderson 1993, 1994). Briefly, to measure phagocytosis, cells were incubated in 96-well microtiter plates at 27°C with FITC-conjugated yeast (50:1 yeast:cell). After 1 h, fluorescence from non-phagocytosed yeast particles was quenched by the addition of trypan blue; the relative phagocytic capacity was then determined by measuring the fluorescent signal from the phagocytosed yeast with a fluorescence concentration analyzer.

Chemiluminescence (CL) measurements were used to assay for O₂⁻ production and H₂O₂-peroxidase-halide activity using the probes lucigenin (bis-

N-methylacridinium nitrate, 10 μM) and luminol (5-amino-2,3-dihydro-1,4-phthal-azinedione, 43 μM), respectively. The resting and phagocytically stimulated (using unopsonized yeast, 20:1 particle:cell) CL responses were measured using a liquid scintillation counter modified for single photon counting.

Quantitation of H_2O_2 was based on the horseradish peroxidase-dependent oxidation of phenol red by H_2O_2 as described by Pick & Mizel (1981). One hour after stimulation with 1 mM phorbol myristate acetate (PMA), the absorbance of oxidized phenol red was measured at 600 nm.

To measure bactericidal activity, phagocytes (2×10^5 per well) were challenged with *Listonella anguillarum* (1.25×10^6 per well). After 3 h, cells were lysed and surviving bacteria allowed to grow for 9 h. Quantitation of bacteria was based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazolium dye that is reduced in direct proportion to the number of viable bacteria present. The extent of cell-mediated killing was determined by comparing the numbers of bacteria in phagocyte-free control wells to those surviving exposure to the phagocytes.

Data from the immunoassays were analyzed using an unpaired Student's *t*-test.

Pentachlorophenol determination. Pronephros, liver and water samples were analyzed for PCP. Pentafluorophenol (PFP) was added to all samples as an internal standard. Extraction and acetylation of PCP from water and tissues were based on the method of Tachikawa et al. (1991). Tissue samples were dissolved overnight in 10 ml of 50% H_2SO_4 . This was extracted with 10 ml of benzene-hexane (1:4). The acid phase was re-extracted 3 times with 5 ml benzene-hexane, and the organic extracts combined. The organic extract was extracted twice with 5% NaOH. The alkaline extract was then acetylated by shaking with 1 ml acetic anhydride for exactly 1 min. The acetylated fraction was extracted twice with either 2.5 or 5 ml *n*-hexane for pronephros or hepatic samples, respectively. Aliquots (10 ml) of water were acidified with 10% H_2SO_4 and extracted twice with 10 ml of hexane. Hexane extracts were combined and extracted twice with 5 ml 5% NaOH. Samples were then acetylated and re-extracted as described for tissue samples.

A Hewlett Packard 5890 Series II gas chromatograph (GC) was used to determine PCP concentrations in tissues and water. The GC was equipped with an electron capture detector (ECD), an electronic pressure controlled split-splitless injector and a DB-5 fused silica capillary column (60 m, 0.320 mm i.d., 0.025 μm film thickness). The GC-ECD temperature program was as follows: 75°C held for 1 min, then increased at a rate of 15°C min^{-1} , and held at a final temperature of 250°C for

3 min. The injector and detector temperatures were 250 and 320°C, respectively. The flow rate of carrier gas (H_2) was 1 ml min^{-1} . Area under the curve of PFP and PCP was integrated using the Hewlett-Packard ChemStation software program; PCP was quantified against a standard curve ranging from 0.0001 to 1 mg l^{-1} PCP.

RESULTS

In both control and treated tanks, fish survival was >96%. The effect of PCP exposure on phagocytic activity by macrophages and eosinophils is shown in Fig. 1. Both cell types ingested FITC-conjugated yeast in the absence of PCP, but their phagocytic activities were not significantly different. Phagocytic capacity of macrophages from PCP-treated fish was not significantly changed; eosinophils had significantly decreased phagocytosis when compared to control fish ($p < 0.05$).

O_2^- and H_2O_2 -peroxidase-halide production were monitored by lucigenin- and luminol-enhanced CL. Three CL parameters were measured: the area under the curve, the peak height, and the background, representing the total stimulated response, maximal stimulated response, and basal or resting activity, respectively. When comparing the responses of macrophages and eosinophils taken from control fish (Figs. 2 & 3), eosinophils had significantly greater stimulated CL responses than the macrophages ($p < 0.005$). PCP treatment produced no significant change in the abilities of either cell type to respond to ROI stimuli, as measured by the 2 CL probes. However, there were significant increases in the background production of

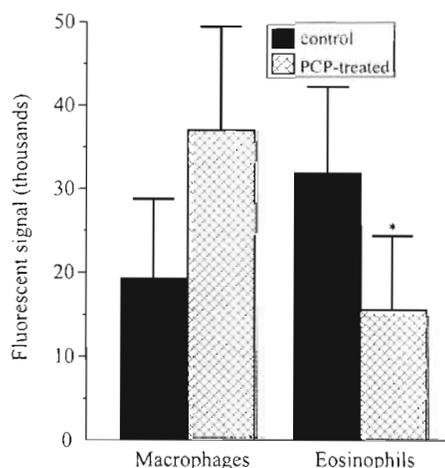


Fig. 1 Effect of PCP on phagocytosis of FITC-conjugated yeast by macrophages and eosinophils. Fluorescent signal of wells with phagocytes and yeast was subtracted from that of control wells with yeast alone. Error bars represent standard error of the mean. * $p < 0.05$

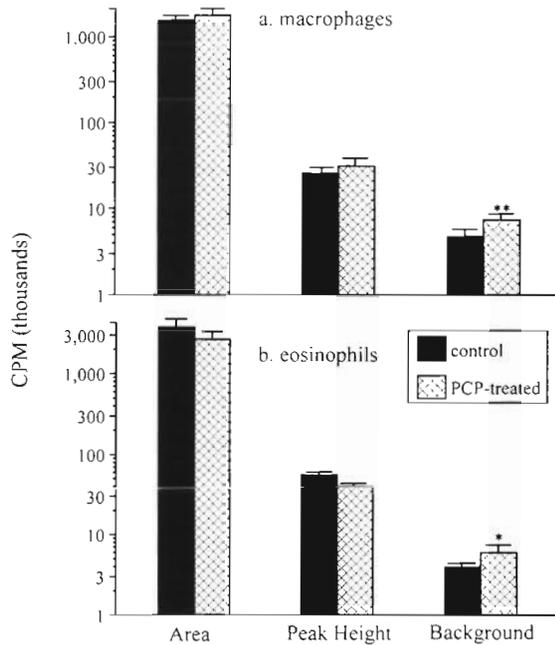


Fig. 2. Effect of PCP on O_2^- production by (a) macrophages and (b) eosinophils, as measured with the chemilumigenic probe lucigenin. Error bars represent standard error of the mean. * $p < 0.05$, ** $p < 0.01$

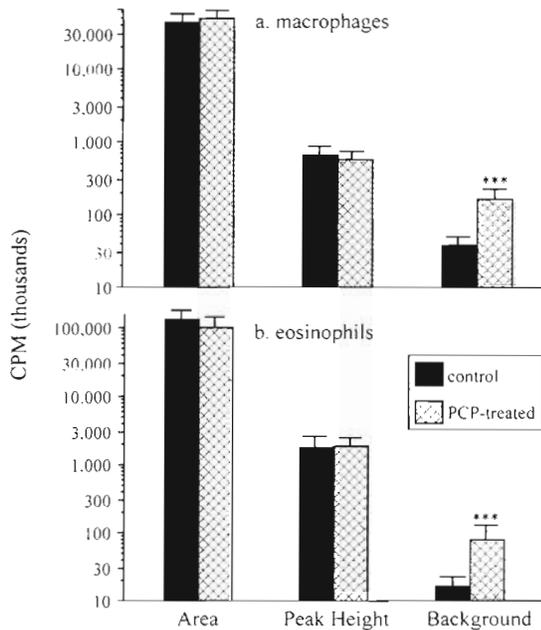


Fig. 3. Effect of PCP on luminol-enhanced chemiluminescence (LCL) of (a) macrophages and (b) eosinophils. LCL is a measure of the H_2O_2 -peroxidase-halide microbicidal system. Error bars represent standard error of the mean. *** $p < 0.001$

both O_2^- and LCL in both cell types after PCP exposure. Production of H_2O_2 by macrophages and eosinophils was not affected by PCP exposure.

Bactericidal activity against *Listonella anguillarum* was significantly inhibited in macrophages ($p < 0.05$) but not in eosinophils taken from PCP-treated fish (Fig. 4). Eosinophils showed significantly greater bactericidal activity than did the macrophages ($p < 0.001$).

Water samples for PCP analysis were taken at the beginning, midway through, and at the termination of the study; tissue samples were taken at the end of the study. At these time points, water concentrations were 0.64 ± 0.31 , 0.73 ± 0.23 , and $0.57 \pm 0.24 \mu g l^{-1}$ in control tanks. In tanks receiving the PCP treatment the concentrations were 37.85 ± 4.30 , 37.57 ± 4.78 and $28.46 \pm 3.28 \mu g l^{-1}$. The concentrations of PCP in the head kidney were 239 ± 50 and $9965 \pm 703 ng g^{-1}$ (wet weight) in control and treated fish, respectively. Liver concentrations of PCP were 120 ± 22 and $9598 \pm 1137 ng g^{-1}$ (wet weight) for control and treated fish.

DISCUSSION

We measured the effects of an environmentally relevant, sublethal, PCP exposure on several non-specific immune functions in an estuarine teleost. Two pronephric cell types were assayed for ROI production and bactericidal activity: macrophages and eosinophils. Concentrations of PCP in the pronephros from treated fish were ~40-fold greater than concentrations in control fish.

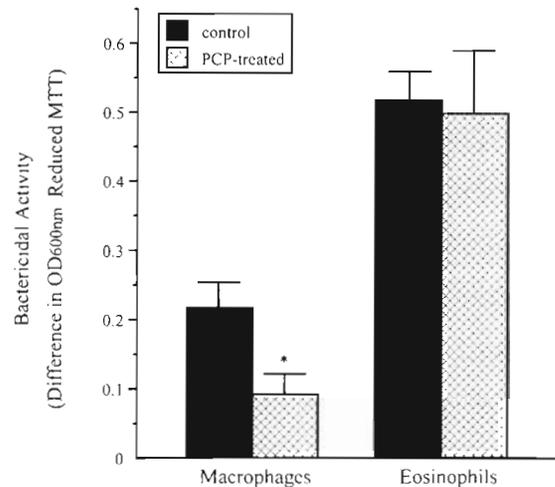


Fig. 4. Effect of PCP on bactericidal activity of macrophages and eosinophils, as measured by the reduction of MTT by viable bacteria. Absorbance of reduced MTT from wells with phagocytes and bacteria was subtracted from the absorbance of wells with bacteria alone. Error bars represent standard error of the mean. * $p < 0.05$

The phagocytic activity of eosinophils was significantly reduced by PCP, but their ability to respond to ROI stimulators and to kill bacteria was apparently not affected by PCP treatment. Several considerations may be relevant when interpreting these results. First, phagocytosis is not an absolute requirement for ROI production; particle contact with the phagocyte can be sufficient. For example, rainbow trout leukocytes were able to generate ROIs in the presence of phagocytic inhibitors following incubation with particulate stimuli (Higson & Jones 1984). Secondly, ROIs and granule-associated cytotoxic proteins produced by eosinophils are frequently expelled into the cytoplasm prior to or following engulfment of target organisms (Klebanoff et al. 1980, 1989). In fish this phenomena has been seen following injection with *Vibrio* and *Aeromonas* extracellular products (Vallejo & Ellis 1989, Powell et al. 1993). This suggested that phagocytosis was not required for bactericidal activity, due to the endocytosis-independent release of ROIs and granule-associated proteins into the extracellular space. Killing observed in these studies might have been mediated by ROIs and granules released after bacteria-leukocyte contact, or by those eosinophils engaged in PCP-insensitive phagocytosis.

In contrast to the eosinophils, phagocytosis by the macrophages was not reduced by exposure of *Fundulus heteroclitus* to PCP. The ability of the macrophages to respond to phagocytic stimuli by producing O_2^- and LCL was also not affected by PCP. However, bactericidal activity was significantly inhibited. Therefore, we must assume that non ROI-mediated mechanism(s) of macrophage-mediated killing was inhibited. This may have been another aspect of free radical-dependent killing, such as nitric oxide (NO) production. Evidence of inducible NO production has recently been described in fish macrophages (to date there is no evidence of inducible NO production in fish granulocytes) (Wang et al. 1995). Decreased bactericidal activity of macrophages taken from PCP-treated fish may also be due to inhibition of non-oxidative microbicidal mechanism(s). Macrophages have cationic proteins with antimicrobial activities, as well as lysosomal enzymes (Beaman & Beaman 1984). Cationic proteins kill both Gram-positive and Gram-negative bacteria via disruption of bacterial membranes. Lysosomal enzymes exert their cidal activity by lysing bacterial cell walls.

In both macrophages and eosinophils, basal levels of O_2^- production and LCL activity were significantly higher after treatment of fish with PCP. The increase in O_2^- production is probably due to PCP's action as an uncoupler of oxidative phosphorylation (Weinbach & Garbus 1965), which results in increased oxygen consumption and an increased rate of electron transport through the hexose monophosphate (HMP) shunt.

NADPH generated by the HMP shunt provides the reducing equivalents for the reduction of oxygen to O_2^- in the respiratory burst. Although respiration rates were not measured in the current experiments, others have found that exposure to PCP led to increases in both respiration and activity of the HMP shunt (Boström & Johansson 1972, McKim et al. 1987), events likely to increase O_2^- production.

The PCP-exposed fish in these experiments showed no overt signs of toxicity. However, the increased basal production of ROIs might overwhelm natural antioxidant defense mechanisms available to the cell, which could in turn lead to ROI-mediated cytotoxicity resulting in enzyme inactivation, membrane destabilization, and/or lipid peroxidation. Increased production of ROIs other than H_2O_2 may also be indirectly responsible for the increased basal levels of LCL. Luminol is typically used to measure H_2O_2 -peroxidase-halide activity in stimulated phagocytic cells (Johansson & Dahlgren 1989, Albrecht & Jungi 1993). However, basal H_2O_2 production was undetectable in our cells, suggesting that the luminol was reacting with another oxidant. During the oxidative burst, luminol has been demonstrated to react with O_2^- , hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2) (Allen 1986, Johansson & Dahlgren 1989); the latter 2 compounds are formed from the reaction of O_2^- with H_2O_2 (Beaman & Beaman 1984). A second possibility is the reaction of luminol with arachidonic acid. Elevated levels of ROIs are likely to cause lipid peroxidation, leading to the release of arachidonic acid from cellular membranes. Arachidonic acid has been shown to generate LCL in both stimulated and unstimulated cells (Andersen et al. 1981; Parnham et al. 1983). Therefore, the higher LCL observed in unstimulated cells may be an indication of lipid peroxidation and arachidonic acid release. In support of this, 2 other compounds which cause oxidative stress, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and endrin, have also been shown to induce O_2^- production and LCL (Numan et al. 1990, Stohs et al. 1990, Bagchi & Stohs 1993).

Exposure of aquatic organisms to pollutants has been linked to increased incidence of disease (Sindermann 1993). Cells of phagocytic lineage such as macrophages and eosinophils represent a first line of defense against harmful bacteria and other potential pathogens. Modulation of their activities through exposure to xenobiotics such as PCP could therefore alter an organism's capacity to resist disease. The PCP-induced changes in immune function seen in these *in vivo* experiments included significantly modulated phagocytosis, ROI levels, and bactericidal activity. Changes such as these could ultimately result in widespread cellular dysfunction and increased incidence of disease.

Acknowledgements. We wish to thank Ms Laurie Mora and Ms Lisa Brubacher for invaluable assistance with the immunoassays. We also thank Dr Joel Baker, Dr John Kucklick, and Mr George Vadas for advice and assistance with the GC-ECD. This work was supported in part by National Institute of Health Training Grant ES07263-05.

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Responsible Subject Editor: D. E. Hinton, Davis, California, USA

Manuscript first received: September 12, 1995
Revised version accepted: March 26, 1996