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IMMUNOMODULATION BY HEAVY METALS TESTED INDIVIDUALLY OR IN MIXTURES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EXPOSED IN VIVO

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Abstract—The objective of this study was to evaluate the effect of heavy metals, at environmentally relevant concentrations, on the immune response of rainbow trout. Trout were exposed for 30 d to cadmium chloride ($CdCl_2$), mercuric chloride ($HgCl_2$), or zinc chloride ($ZnCl_2$) either individually or in combinations: $CdCl_2/HgCl_2$, $CdCl_2/ZnCl_2$, $HgCl_2/ZnCl_2$, or $CdCl_2/HgCl_2/ZnCl_2$. Following the 30-d exposure, parameters of the nonspecific cellular immune response (phagocytosis, respiratory burst, and lymphoblastic proliferation) and of the nonspecific humoral immune response (lysozyme activity and the level of immunoglobulin) were measured. The results obtained indicate that individually, all three metals tested induce significant immunomodulations. However, the toxicity of mercury or cadmium is significantly reduced in fish simultaneously exposed to zinc, indicating that a protection is afforded by zinc against cadmium- and mercury-induced immunotoxicity.

Keywords—Immune response Heavy metals Rainbow trout Cytometry

INTRODUCTION

In their environment, feral fish are subjected to xenobiotics that are capable of altering various physiological parameters, such as the reproductive, nervous, endocrine, and immune systems. Heavy metals, namely mercury, cadmium, and zinc, are important pollutants of aquatic systems and have been shown to alter the immune response of several fish species [1,2]. The main function of the immune system is to protect the organism against potentially harmful agents (bacteria, viruses, parasites) through a delicate balance of immunocompetent cell differentiation and proliferation. When subjected to toxic substances, components of the immune system may be altered, thereby affecting the balance-regulating immune functions and, in turn, causing immunopotentiation (autoimmune diseases) or immunodepression (increased susceptibility to pathogens). These alterations may weaken the animal, and in more severe exposures, may cause death [3].

The immunomodulatory effects induced in vertebrates, including fish, by mercury [4,5] and cadmium [6,7] are well documented. Zinc is an essential trace element and can cause growth retardation, histopathological alterations, and death when present in high concentrations. In contrast to terrestrial animals, fish are often exposed to elevated concentrations of zinc, and an altered immune response in fish exposed to the metal has been observed [8,9]. Furthermore, zinc is known to reduce the toxicity of cadmium and mercury through the increased production of metallothionein (MT), a metal-binding protein.

In aquatic environments, animals are subjected to a mixture rather than to individual contaminants, but very few studies have investigated the combined effects of heavy metals on the immune response. The objective of the present study is to characterize the effects of cadmium, mercury, and zinc, alone or in combination, on the immune response of rainbow trout (*Oncorhynchus mykiss*) exposed in vivo to environmentally relevant concentrations of metals. Following a 30-d exposure period, parameters of the nonspecific and specific immune response were evaluated.

MATERIAL AND METHODS

Experimental design

Juvenile rainbow trout (Walbaum), weighing on average 100 g, were obtained from a local commercial hatchery and were acclimated for 2 weeks in aerated, chlorine-free tap water at a temperature of 15°C. They were fed daily with a ration equal to 1% body weight and were maintained on a photoperiod of 12-h day/night. Groups of 12 rainbow trout were exposed to various concentrations of cadmium chloride ($CdCl_2$), mercuric chloride ($HgCl_2$), or zinc chloride ($ZnCl_2$), individually or in combinations (Table 1).

The chemicals, $HgCl_2$ (ultrapure grade, 99.999%), $ZnCl_2$ (ultrapure grade, 99.999%), and $CdCl_2$ (ultrapure grade, 99.99%), were purchased from Aldrich Chemical (Milwaukee, WI, USA). The cadmium, mercury, and zinc concentrations were maintained in the tanks at a constant level with Mariotte

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Table 1. Combinations and concentrations of metals tested in vivo on rainbow trout[a]

		$HgCl_2$			$CdCl_2$			$ZnCl_2$		
		0.1 μg/L	0.3 μg/L	0.5 μg/L	1 μg/L	3 μg/L	5 μg/L	10 μg/L	30 μg/L	50 μg/L
$HgCl_2$	0.1 μg/L	++								
	0.3 μg/L		++			++				
	0.5 μg/L			++			++			
$CdCl_2$	1 μg/L				++					
	3 μg/L		++				++			
	5 μg/L									
$ZnCl_2$	10 μg/L							++		
	30 μg/L		++			++				
	50 μg/L			++			++			++
$HgCl_2$ + $ZnCl_2$	0.3μg/L + 30 μg/L					++				
	0.5 μg/L + 50 μg/L						++			

[a] $HgCl_2$ = mercuric chloride; $CdCl_2$ = cadmium chloride; $ZnCl_2$ = zinc chloride.

bottles [10]. Previous work by Hontela et al. [11] on cadmium and mercury showed that with this technique, fish were exposed similarly, in the water throughout the tank, to the metals. After 30 d, the fish were anesthetized with 0.3 ml/L of 2-phenoxyethanol (1.109 g/ml; ICN Laboratory, Costa Mesa, CA, USA) and were then euthanized. The blood was collected with a heparinized syringe from the caudal peduncle. The thymus and the head kidney were removed, and a single cell suspension was prepared under sterile conditions by grinding the tissues on a metal mesh with cold Hank's balanced salt solution (HBSS) medium (Gibco, Gaithersburg, MD, USA) supplemented with 10 U/ml of heparin, 1% solution of penicillin (100 units/ml), and streptomycin (100 mg/ml) (Gibco). The suspensions were then placed over a Ficoll gradient (d = 1.077g/ml; Pharmacia, Uppsala, Sweden) and spun 20 min at 1000 *g* to remove erythrocytes and debris. The cells located at the interface of the medium and the Ficoll were then collected and washed once in cold HBSS. The pellet was suspended in RPMI-1640 (Gibco) medium, and the cell and viability counts were performed using trypan blue dye exclusion test. Plasma was obtained by centrifuging the blood at 400 *g* for 20 min followed by storage at −20°C until analysis.

Phagocytosis

The phagocytic activity of head-kidney macrophages was determined by flow cytometric evaluation of cells engulfing fluorescent latex microspheres (diameter = 1.72 μm; Polysciences, Warrington, PA, USA), as described by Voccia et al. [7].

Respiratory burst

The production of hydrogen peroxide (H_2O_2) was measured and modified by Voccia et al. [7] for flow cytometry.

Lymphoblastic proliferation

Proliferation of head-kidney and thymic leukocytes was evaluated as described by Voccia et al. [7].

Plasma immunoglobulin M measurement

The plasma concentration of immunoglobulins was determined as described by Thuvander et al. [12] and as modified in our laboratory. An ELISA procedure was used to test the reactivity of a monoclonal antibody with immunoglobulin M (IgM) from rainbow trout. A volume of 100 μl of goat anti-mouse (Gibco), diluted 1:1,000, was added to the wells of polystyrene microtitre plates. The plates were incubated overnight at room temperature. All the tests were made in triplicate. After each incubation, the plates were washed twice with PBS containing 0.2% Tween 20 (Fisher, Nepean, ON, Canada). A volume of 100 μl of mouse monoclonal anti-trout Ig 1-14 (DeLuca et al. [13]) was added to each well, and plates were incubated 1 h at room temperature, incubation was followed by the addition of 100 μl of fish serum diluted in PBS-Tween (2:500). The plates were incubated overnight at 4°C; in negative control cultures, PBS was added. Finally, 100 μl of peroxidase-labeled affinity-purified antibody to trout immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well and incubated for 1 h at room temperature. After the last wash, 100 μl of freshly mixed peroxidase substrate solution (ABTS, Kirkegaard and Perry Laboratories) was added to each well. The reaction was stopped after 10 min with 100 μl of a stop solution (Kirkegaard and Perry Laboratories), and the absorbency was read with an ELISA reader (Multiskan MCC/340 MTX Lab Systems, McLean, VA, USA) at a wavelength between 405 and 410 nm.

Lysozyme activity

In a 96-well ELISA plate, 10-μl aliquots of serum and 100 μl of *Micrococcus lysodeikticus* (0.15 mg/ml) were placed in triplicate. The optical densities were read at 450 nm after 30 min. The results were expressed as mean slopes, which represented the lysis of the bacterial suspension by lysozymes over time.

Statistics

The data were first tested for normality and homogeneity with the Bartlett's test. Since all data were normal, we then determined, using an analysis of variance, whether there was a difference between groups, and if so, the Tukey test was performed (Toxstat® 3.2, Statistics Unlimited, Westford, MA, USA). However, when the data set had unequal replicates, the Bonferonni *t* test was used [14].

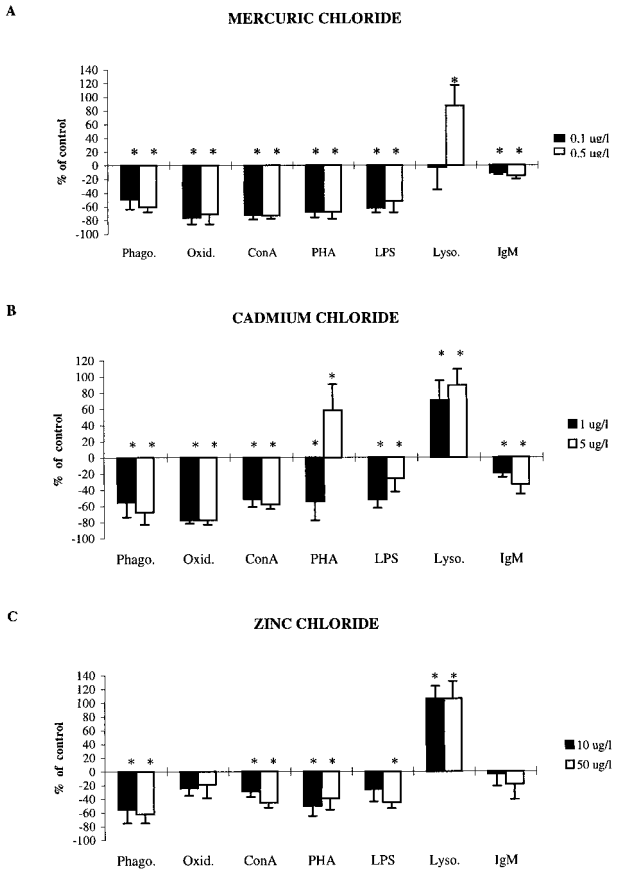

Fig. 1. Effects of a 30-d exposure to mercuric chloride ($HgCl_2$; 0.1 μg/L and 0.5 μg/L) (**A**), cadmium chloride ($CdCl_2$; 1 μg/L and 5 μg/L) (**B**), and zinc chloride ($ZnCl_2$; 10 μg/L and 50 μg/L) (**C**) on the rainbow trout immune response. Phago. = phagocytosis; Oxid. = oxidative burst; Con A (thymus, 10 μg/ml); PHA (head kidney, 20 μg/ml); LPS (head kidney, 100 μg/ml); Lyso. = lysozyme. The results are expressed as a percentage compared with control. * $p < 0.05$ (significantly different from the control).

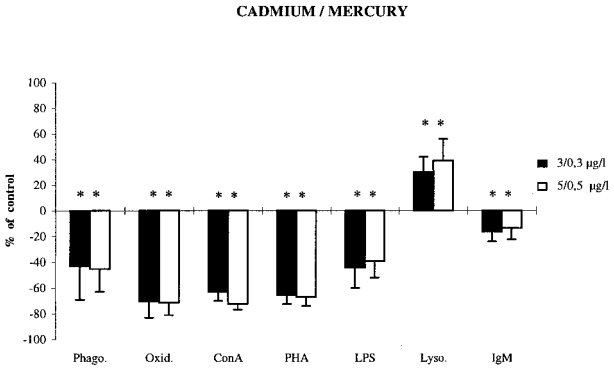

Fig. 2. Effects of cadmium chloride ($CdCl_2$) and mercuric chloride ($HgCl_2$) mixtures on the immune system of rainbow trout following a 5-week exposure to 3 μg/L and 0.3 μg/L of cadmium and mercuric chloride, respectively, and 5 μg/L and 0.5 μg/L of cadmium and mercuric chloride, respectively. Phago. = phagocytosis; Oxid. = oxidative burst; Con A (thymus, 10 μg/ml); PHA (head kidney, 20 μg/ml); LPS (head kidney, 100 μg/ml); Lyso. = lysozyme. The results are expressed as a percentage compared with control. * $p < 0.05$ (significantly different from control responses).

RESULTS

Effects of individual metals on the immune response of rainbow trout

The in vivo effects of $HgCl_2$, $CdCl_2$, and $ZnCl_2$ on the immune competence of rainbow trout were evaluated in the first part of this study. The results are presented in Figure 1A, B, and C.

Individual exposures to $HgCl_2$ (0.1 and 0.5 μg/L), $CdCl_2$ (1 and 5 μg/L), or $ZnCl_2$ (10 and 50 μg/L) significantly inhibited the phagocytosis of latex beads by head-kidney macrophages. Exposure to $HgCl_2$ and $CdCl_2$ also caused a severe inhibition of the oxidative burst in the same cells, whereas an exposure to $ZnCl_2$ had no significant effect on the production of H_2O_2.

The lysozyme activity, a nonspecific humoral factor, was stimulated in fish exposed to both concentrations of cadmium and zinc but was stimulated only at the highest concentration of mercury. The proliferation of thymic lymphocytes stimulated with Concanavalin A (ConA) was significantly inhibited in fish exposed to both concentrations of all three metals, with the following order of toxicity: $HgCl_2 > CdCl_2 > ZnCl_2$. Lymphocytes from the head kidney of trout exposed to mercury had a lower proliferative response following a stimulation with phytohemagglutinin (PHA) or lipopolysaccharides (LPS); the inhibition was similar to that observed with thymocytes. In fish exposed to $ZnCl_2$, the proliferation induced by PHA was significantly inhibited at both concentrations tested, whereas only the highest concentration significantly inhibited the response to LPS. In the case of $CdCl_2$, an exposure to 1 μg/L inhibited the proliferative response of head-kidney lymphocytes to PHA, but a stimulation was observed at 5 μg/L. Both concentrations of $CdCl_2$ tested inhibited the proliferative response of head-kidney lymphocytes with LPS.

Effects of metal mixtures on the immune response of rainbow trout

In the second part of this study, rainbow trout were exposed to combinations of metals. When cadmium was mixed with mercury (Fig. 2), phagocytosis and the respiratory burst by macrophages were significantly inhibited. However, lysozyme activity, another parameter of the nonspecific immune response, was stimulated. The intensity of the responses was similar to those seen when both metals were tested individually.

The proliferative response of thymocytes and of head-kidney lymphocytes was significantly inhibited in fish exposed to both metals, and the pattern of inhibition resembled the effect of mercury, with the response to LPS being the least affected. When zinc was mixed with either cadmium (Fig. 3) or mercury (Fig. 4), a significant reduction in the immunotoxicity of these two metals was observed. For cadmium, zinc significantly reduced the toxicity of cadmium on the nonspecific immune response: macrophage activity (phagocytosis and respiratory burst) and lysozyme activity. However, the proliferative response of thymocytes and head-kidney lymphocytes as well as the level of Ig were still significantly lower than those of control fish. In the case of mercury, zinc significantly reduced the toxicity of mercury on the nonspecific immune response, except for the respiratory burst of macrophages in fish exposed to the highest concentration. Zinc also reduced the inhibition of mercury on the proliferative response of thymocytes but not that of head-kidney lymphocytes. Finally, when zinc was mixed with cadmium and mercury (Fig. 5), the

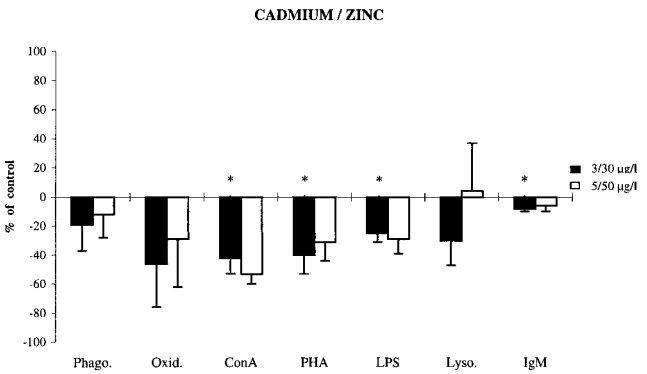

Fig. 3. Effects of cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$) mixtures on parameters of the immune system of rainbow trout following a 5-week exposure to 3 μg/L or 30 μg/L of cadmium and zinc chloride, respectively, and 5 μg/L or 50 μg/L of cadmium and zinc chloride, respectively. Phago. = phagocytosis; Oxid. = oxidative burst; Con A (thymus, 10 μg/ml); PHA (head kidney, 20 μg/ml); LPS (head kidney, 100 μg/ml); Lyso. = lysozyme. The results are expressed as a percentage compared with control. * $p < 0.05$ (significantly different from control responses).

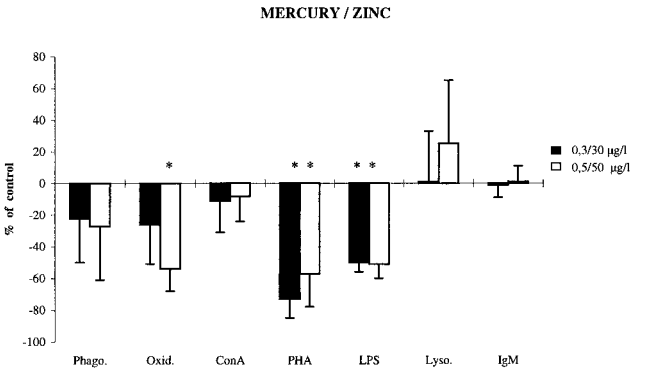

Fig. 4. Effects of mercuric chloride ($HgCl_2$) and zinc chloride ($ZnCl_2$) mixtures on the immune system of rainbow trout exposed for 5 weeks to 0.3 μg/L or 30 μg/L of mercuric chloride and zinc chloride, respectively, and 0.5 μg/L or 50 μg/L of mercuric chloride and zinc chloride, respectively. Phago. = phagocytosis; Oxid = oxidative burst; Con A (thymus, 10 μg/ml); PHA (head kidney, 20 μg/ml); LPS (head kidney, 100 μg/ml); Lyso. = lysozyme. The results are expressed as a percentage of control. * $p < 0.05$ (significantly different from control responses).

protective effect of zinc was less important, since all parameters tested were significantly different from the controls. Results from these studies show that zinc reduces the toxic effect of cadmium and mercury on the immune response of rainbow trout.

DISCUSSION

The objective of this study was to determine the effects of $CdCl_2$, $HgCl_2$, and $ZnCl_2$, individually and in combinations, on the immune response of rainbow trout. Several parameters were tested in order to evaluate the possible effects on humoral factors, such as lysozyme activity and the level of Ig, as well as the effects on cellular functions, such as phagocytosis and lymphoproliferation.

The first part of this study consisted of evaluating the immune response of rainbow trout exposed for 30 d to $CdCl_2$, $HgCl_2$, or $ZnCl_2$. Our results demonstrated that at environmentally relevant concentrations, these three metals significantly modulate the immune response of rainbow trout, since the majority of the parameters reflecting cellular and humoral responses were affected. Overall, the results indicate an immunosuppression. Studies depicting an immunosuppression induced by an exposure to either mercury [4,5,15], cadmium [1,7,16–18], or zinc [8,19,20] are numerous in fish and mammals. However, conflicting results are seen in some species, indicating a difference in sensitivity according to the species tested, the concentrations used, and the time and/or exposure route.

It is unlikely that a single mechanism can account for the immunotoxic effects of all heavy metals. These metals can affect immunocompetent cells in various ways; for instance, mercury is known to disrupt microtubules [21], to increase the intracellular concentration of calcium [22], to decrease the production of glutathione [23], and to induce apoptosis [24]. As for cadmium, it competes with calcium for binding sites essential to the induction of cellular proliferation [25], it induces apoptosis [26,27], it inhibits proliferation by decreasing the number of cells in S and G2-M phases [28], and it affects potassium currents in B lymphocytes [29]. Zinc is an essential metal that acts as a membrane stabilizer by binding structural proteins and is an essential cofactor in several metalloenzymes (DNA polymerase, collagenase, superoxide dismutase). A deficiency in zinc is apparent in people with a congenital deficiency for a zinc-dependent enzyme (nucleoside phosphorylase, Npase); these individuals experience problems with their cell-mediated immunity [30].

In the second part of this study, we further investigated the effect of mixtures of metals on fish immune functions. The results obtained show that when trout were exposed to cadmium and zinc or to mercury and zinc, the observed immunosuppression is less severe than that of fish that were exposed to the metals individually. In the case of cadmium, the nonspecific immune parameters (phagocytosis, oxidative burst, and lysozyme activity) were no longer significantly inhibited. For mercury, humoral parameters (Ig level and lysozyme activity), phagocyte activity, and the proliferative response of thymocytes were no longer significantly affected. That zinc protects cadmium- or mercury-induced toxicity has been

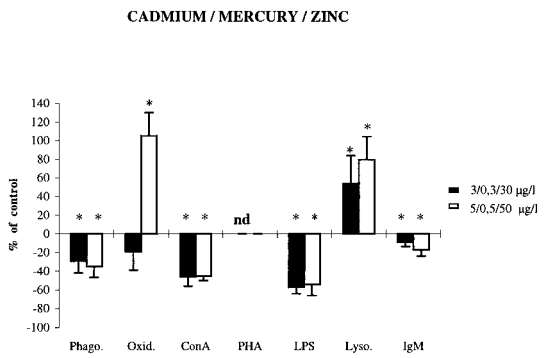

Fig. 5. Effects of cadmium chloride ($CdCl_2$), mercuric chloride ($HgCl_2$), and zinc chloride ($ZnCl_2$) mixtures on the immune system of rainbow trout following a 5-week exposure to 3 μg/L, 0.3 μg/L, and 30 μg/L of cadmium, mercuric, and zinc chloride, respectively, and 5 μg/L, 0.5 μg/L, and 50 μg/L of cadmium, mercuric, and zinc chloride, respectively. Phago. = phagocytosis; Oxid. = oxidative burst; Con A (thymus, 10 μg/ml); PHA (head kidney, 20 μg/ml); LPS (head kidney, 100 μg/ml); Lyso. = lysozyme. The results are expressed as a percentage compared with control. * $p < 0.05$ (significantly different from control responses).

shown in several toxicological [27,31,32] and immunotoxicological studies [33,34].

Zinc can protect cells from cadmium-induced toxicity by (1) inducing the synthesis of metallothionein, a metal protein complex that sequesters and reduces the amount of free metal in the tissues, thereby reducing their potential toxicity; (2) preventing the entry of cadmium into the cell; and (3) competing with cadmium for intracellular binding sites [32]. The induction of metallothionein and glutathione by cadmium is probably one of the most important mechanisms of cellular protection against this metal. In mammalian cell systems, it has been shown that cells exhibiting a higher level of metallothionein are less sensitive to cadmium [35–37] or mercury [31] toxicity.

Rainbow trout also possess metallothionein, with zinc being more effective than cadmium in inducing metallothionein mRNA [38]. As with mammals, a preexposure of rainbow trout to zinc diminishes the fish's sensitivity to cadmium [39]. Although the exact mechanism(s) involved in the toxicity of heavy metals on trout immune response has not been established yet, our results show that rainbow trout are sensitive to heavy metal exposure. Furthermore, this study demonstrated that zinc protects trout against mercury and cadmium toxicity and that the cellular mechanisms involved may be similar to those characterized in mammals.

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