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Non-specific immune response of Zebrafish, *Brachydanio rerio* (Hamilton–Buchanan) following copper and zinc exposure

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The cellularity of the kidney and its non-specific immune function, as assessed by macrophage phagocytosis and spontaneous natural cytotoxicity, was studied in *Brachydanio rerio* kidney cells following sublethal *in vivo* and *in vitro* exposure to different copper and zinc concentrations. *In vivo* effects of heavy metals were studied in fish exposed to 0·05, 0·15 and 0·25 mg l^{-1} zinc and 0·05, 0·10 and 0·15 mg l^{-1} copper during 7 days at 22° C. Copper caused a dose-dependent decrease in the number of kidney leucocytes, NCC activity and phagocytic response. Fish exposed to zinc concentrations had a lower cytotoxic activity, an enhanced macrophage response and lower counts of kidney lymphocytes when compared to the control group. *In vitro* exposure of leucocytes to copper at 5, 10 and 20 μg ml^{-1} or to zinc at 10, 50 and 100 μg ml^{-1} for 1 h showed the same immunomodulatory effects as seen in the *in vivo* exposure.

Key words: *Brachydanio rerio*, heavy metals, immunotoxicity, non-specific cytotoxic cell activity, phagocytosis.

I. Introduction

Industrial discharges, run-off from farms, city effluents and other contaminants of human origin are important pollutants present in the aquatic environment and thus unavoidable by fish (Cross *et al.*, 1985). Environmental stressors, especially pollutants, have been shown to be coincident with the outbreak of infectious diseases in fish, many examples of which have been reviewed by Snieszko (1974). The modulation of the immune system of fish may result from the action of pollutants in the environment on critical physiological pathways.

Sublethal exposure to copper suppressed fish resistance to viral (Hetrick *et al.*, 1979) and bacterial pathogens (Baker & Knittel, 1983; Rougier *et al.*, 1992). Depending on the immune parameters measured, such as humoral or cellular immunity, both suppressive and stimulatory effects of zinc have been reported (Ghanmi *et al.*, 1989).

Fish macrophages comprise an important part of the cellular immune system and function to protect the host by phagocytosing invading microorganisms as

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they enter the tissues. Several studies have shown that exposure of fish to heavy metals modulated macrophage function. Heavy metals such as copper (Kangarot & Tripathi, 1991), cadmium (Elsasser *et al.*, 1986) and manganese (Cossarini-Dunier *et al.*, 1988) cause both suppressive and stimulatory effects on fish macrophage activity. Spontaneous natural cytotoxicity is an important cytolytic effector mechanism in resistance to tumours and viral diseases in mammals. Many heavy metals have proved to modulate this activity following acute or chronic exposure (Descotes, 1986). Studies of lower vertebrates have shown that fresh water fish may have cytotoxic cells with a non-specific lytic activity against certain transformed and established mammalian cell lines (Deschaux *et al.*, 1983; Greenlee *et al.*, 1991). The non-specific cytotoxic cell (NCC) has been found in several species of fish and was identified as monocyte-like (Evans *et al.*, 1984*a*). However very little is known of the toxicity of heavy metals on natural cytotoxicity in fish.

The present approach was stimulated by the suggestion that the zebrafish, *Brachydanio rerio*, may be a model for aquatic immunotoxicological studies. This fish has often been used to assess the toxicity of chemical products (Devillers *et al.*, 1985). We chose this species to examine both *in vivo* and *in vitro* effects of Zn^{2+} and Cu^{2+} on fish non-specific immune response.

II. Materials and Methods

FISH HOLDING AND METAL EXPOSURE

Zebrafish, *Brachydanio rerio* (Hamilton–Buchanan), measuring 2–4 cm and weighing 0·3–0·5 g were maintained in 60-l glass aquaria for 15 days before metal exposure. During acclimation fish were maintained at $22 \pm 1°$ C, pH $6{\cdot}7 \pm 0{\cdot}1$ and fed every day with commercial food (Tetramin). For the exposure period, groups of 30 fish were kept in 8-l glass aquaria for 7 days at $22 \pm 1°$ C and the water was not changed. Fish were not fed 24 h before immunological experimentation [AFNOR (Association Française de Normalisation), 1978]. Reagent grade cupric chloride ($CuCl_2$, $2H_2O$) and zinc chloride ($ZnCl_2$) was used to prepare test concentrations. Test fish were exposed to three copper and zinc concentrations (0·05, 0·15 and 0·25 mg l^{-1} for zinc and 0·05, 0·10 and 0·15 mg l^{-1} for copper). The physicochemical properties of the test water were determined and the metal concentration in the water was checked by atomic absorption methods. Three replicate samples were used for each copper and zinc concentration. Controls without added metals were run under similar conditions.

PREPARATION OF LYMPHOID CELL SUSPENSION

After 7 days of metal exposure, 30 fish were used for the preparation of lymphoid cell suspensions. The pro and mesonephric kidney from 30 fish were pooled in to 3 ml RPMI-1640 Dutch modification (041-02409 M: GIBCO, Life Technologies Ltd., Paisley, Scotland). The culture medium contained heat inactivated foetal calf serum (FCS) (Labsystems-Flow, Les Ulis, France) (10%) and was supplemented with a mixture of penicillin (100 UI ml^{-1}) and streptomycin (100 μg ml^{-1}). To form a single-cell suspension, the organs were teased

and filtered through a sterile membrane (20 μm) (Scrynel, Filtre durieux, France). Kidney lymphoid cells were obtained by centrifugation (400 *g* for 25 min) over Isopaque-Ficoll (1·077 gcm^{-3}). The lymphoid cell suspension was then centrifuged at room temperature for 10 min at 400 *g* and washed twice in 5 ml RPMI-1640. Lymphoid cells in the suspension were counted using a haemocytometer, the cell viability was determined by the trypan blue exclusion method and the cell suspension adjusted to 5×10^6 viable cells ml^{-1}.

TUMOUR TARGET CELLS

The mammalian P815 cell line (Hôpital Léon Bérard, Lyon, France) from mice DBA/2 was maintained at 37° C in a 95% air/5% CO_2 humidified atmosphere. The culture medium, RPMI-1640 contained FCS (10%) and antibiotic solution [penicillin (100 UI ml^{-1})/streptomycin (100 μmg ml^{-1})]. Viability of cells was tested with trypan blue, 95% viability being acceptable.

CYTOTOXICITY

One million target cells were labelled with 0·2 mCi of ^{51}Cr-Na_2CrO_4 (Amersham, France) for 1 h at 37° C. Cells were washed three times in RPMI-1640, diluted to 5×10^4 cells ml^{-1} and 5×10^3 cells in 100 μl were plotted into a 96-well round-bottom tissue culture plate (Nunc, Polylabo, France). Effector cells were then added to 100-μl aliquot volumes at 100:1, 50:1 and 25:1 effector to target (E:T) cell ratios. The plates were centrifuged (50 *g*) for 5 min and incubated at 28° C. After 6 h of incubation, supernatants were harvested and radioactivity was determined in a Packard Cobra 5000 Gamma counter. Percent specific release was calculated as follows:

$$\% \text{ specific release} = \frac{(\text{test release}) - (\text{spontaneous release})}{(\text{total release}) - (\text{spontaneous release})} \times 100 \quad (1)$$

PREPARATION OF KIDNEY PHAGOCYTES

Fish from the control group and those exposed *in vivo* to different concentrations of zinc and copper for 7 days were used for this study. Kidney tissue was removed from 30 fish, teased out in 3 ml of RPMI-1640 containing FCS (2%), penicillin/streptomycin and 5 IU ml^{-1} heparin and filtered through sterile membrane (20 μm) to remove large tissue fragments. Lymphoid cell suspensions were enriched for monocytes by centrifugation over 34%/51% v/v Percoll (Pharmacia, France) discontinuous density gradients. The Percoll solution was diluted directly with distilled water, using × 10 Hank's balanced salt solution (HBSS) to make it isosmotic and 5N HCl to adjust the pH to 7·6. The gradient was then centrifuged at 400 *g* for 25 min at 4° C, and the band of monocyte cells was collected at the 34–51% interface. The cells in the suspension were washed twice in 5 ml RPMI-1640, counted, cell viability was determined by the trypan blue exclusion method and adjusted to a final concentration of 2×10^5 cells ml^{-1} with RPMI-1640.

BACTERIA

Aeromonas hydrophila (95314) was isolated from *B. rerio* intestines. Bacterial cultures were grown in sterile trypticase® soy agar (TSA) (Laboratoire Départemental d'Analyses et de Recherches, Limoges, France) at 37° C for 24 h. Heat killed bacteria were obtained by incubating live microorganisms at 60° C for 45 min. Aliquots were stored at 4° C.

PHAGOCYTOSIS ASSAY

Five-hundred microlitres of cell suspension (10^5 cells) was delivered into each well of a 6-well plastic tissue culture plate (Nunc, Polylabo, France) and incubated at 28° C for 1 h (adherence period). To all wells, 1 ml of heat killed bacterial solution (2×10^7 ml^{-1}) in RPMI-1640 (FCS 2%, penicillin/streptomycin) was added and incubated for 180 min at room temperature (22° C). Wells were gently rinsed in RPMI-1640 and stained with Giemsa solution. One hundred macrophages were counted per well and the number of macrophage cells containing bacteria, as well as the number of bacteria per phagocyte, were counted.

IN VITRO HEAVY METALS LYMPHOID CELL TREATMENT

A stock solution of copper chloride and zinc chloride was made with sterile distilled water so that the resultant metal concentration was 10 mg ml^{-1}. Serial dilutions were made in RPMI-1640 for the culture of cells (pH 7·3 ± 0·1). Final concentrations were 20, 10, 5 and 0 μg ml^{-1} for the copper treatment and 100, 50, 10 and 0 μg ml^{-1} for the zinc treatment. Three replicates were used for each copper and zinc concentration. The phagocytes were treated 1 h at 28° C during the adherence period. The lymphocytes used in the cytotoxicity test were treated with the heavy metal solutions at 28° C. After 1 h, the lymphoid cells were washed twice in RPMI-1640, counted, cell viability determined and adjusted to a final concentration of 5×10^6 viable cells/ml.

STATISTICAL ANALYSIS

Differences between groups of means of control and metal treated fish or cells were analysed by Student's *t*-test. Representative data (mean ± S.E.M.) from triplicate experiments of 30 fish pooled in each experiment.

III. Results

Throughout the experiment, fish from control groups and those exposed to the metals showed normal behaviour and remained clinically healthy. The *in vivo* concentrations of the heavy metals at the end of exposure (7 days) were within ± 50% of its initial concentrations.

CELLULARITY OF LYMPHOID ORGAN

The total number of kidney cells from 30 individuals was determined in control and metal-treated fish after 7 days of exposure. At 0·05 mg ml^{-1} of

Table 1. Effect of copper and zinc exposure on the number of kidney leucocytes of *B. rerio* after 7 days of exposure

Metal	Concentration (mg l^{-1})	No. of lymphoid cells $\times 10^6$
Control	0	9·45 ± 0·86
Zinc	0·05	6·95 ± 0·45†
	0·15	7·15 ± 0·79
	0·25	7·65 ± 0·68
Copper	0·05	6·48 ± 0·48†
	0·10	6·22 ± 0·52†
	0·15	5·78 ± 0·40‡

*Representative data (mean ± S.E.M.) from triplicate experiments of 30 fish pooled in each experiment.
†$P<0·05$.
‡$P<0·01$.

Table 2. The NCC activity of *B. rerio* kidney cells was measured after 6, 18 and 24 h incubation at 28° C. Target cells (P815) were mixed with fish kidney cells at three, Target cells:Effector cells (T:E) ratios. Data are representative from triplicate experiments, mean ± S.E.M. of 90 fish

Incubation (h)	Percentage of ^{51}Cr specific release		
	1:100	1:50	1:25
6	25·6 ± 2·4	11·8 ± 2·4	3·9 ± 1·6
18	33·9 ± 4·1	20·7 ± 3·8	12·3 ± 3·8
24	49·0 ± 16·2	30·9 ± 3·4	20·1 ± 4·5

zinc, the proportion of leucocytes was significantly decreased ($P<0·05$) from those of the control group. No significant difference was observed in fish treated with 0·25 and 0·15 mg l^{-1} of zinc and the control group. The level of cells was markedly reduced in copper-treated groups (0·05, 0·10 and 0·15 mg l^{-1}) as compared to the control group (Table 1).

IN VIVO AND *IN VITRO* EFFECTS OF HEAVY METALS ON CYTOTOXICITY

Kidney cells from both control and metal-exposed fish were tested for cytolytic activity against P815 chromium 51 labelled target cells. In control fish, cytotoxic activity occurred after 6 h and increased after 18 and 24 h incubation (Table 2). The NCC activity of fish kidney cells was altered by sublethal exposure to zinc and coper (Fig. 1). At 0·15 and 0·25 mg l^{-1} of zinc the cytotoxicity was significantly decreased but only at the optimum killing ratio (1:100). At different E:T ratios no significant differences in cytotoxicity responses were observed between control and zinc-exposed fish at 0·05 mg l^{-1}. In contrast, at 0·05 mg l^{-1} of copper (Fig. 1) the cytotoxicity activity of the exposed fish kidney cells was significantly decreased for 1:100 ratio as compared to the control group. At 0·10 mg l^{-1} of copper, the NCC activity was

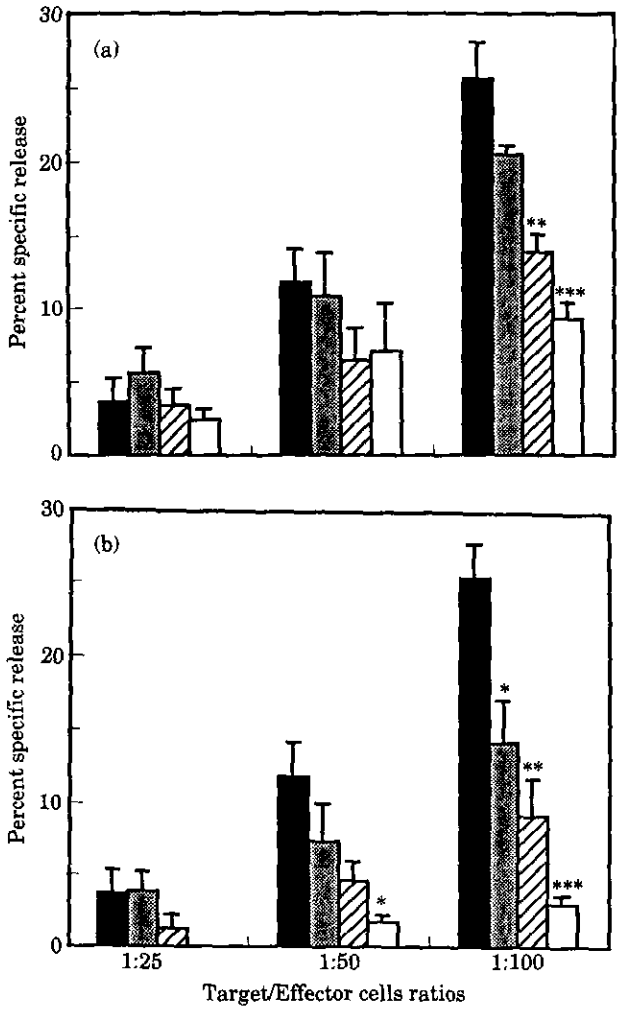

Fig. 1. B. rerio were exposed to (a) three zinc and (b) three copper concentrations during 7 days at 22° C (■) Control, (▩) 0·05 mg l^{-1}, (▨) 0·10 mg l^{-1}, (□) 0·15 mg l^{-1}. Kidney cells from both control and metal-exposed fish were tested for cytolytic activity against P815 chromium^{51} labelled target cells. The NCC activity of fish kidneys cells was measured after 6 h incubation at 28° C. Target cells were mixed with kidney cells at indicated T:E ratios. Representative data from triplicate experiments of 30 fish pooled in each experiment. Significant differences with respect to value for control at $*P<0·05$, $**P<0·01$ and $***P<0·001$, respectively (Student's *t*-test).

decreased only for 1:100 ratio and at 0·15 mg l^{-1} this activity was significantly decreased for 1:50 and 1:100 ratios. The *in vitro* effect of heavy metals on the NCC cytolytic response was also determined. Kidney cells (effector cells) were first preincubated in culture medium containing different concentrations of zinc and copper for 1 h at 28° C. No significant change was observed in the cell viability of kidney cells at three concentrations of zinc and copper as

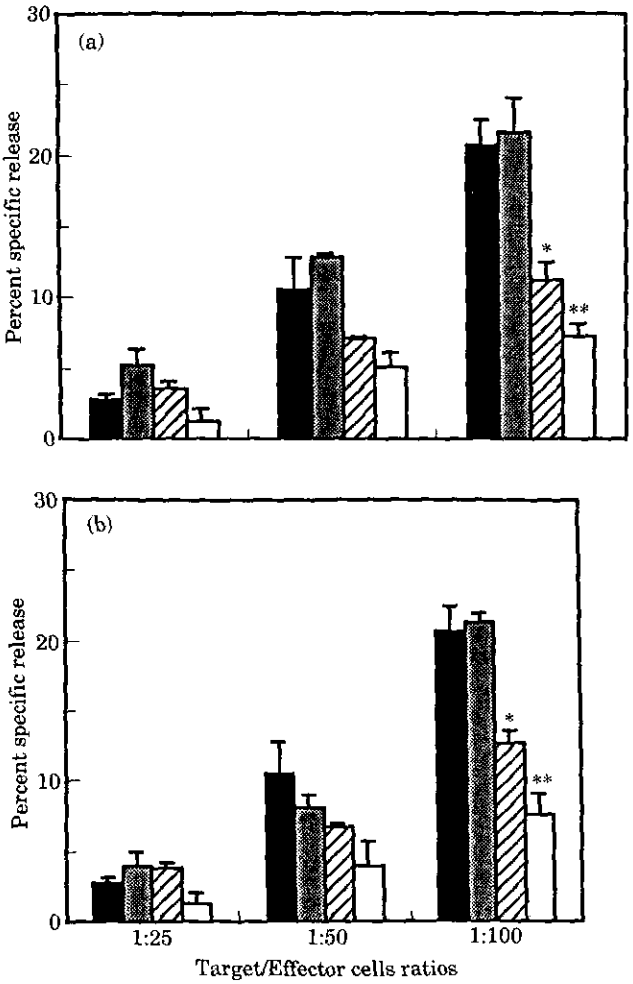

Fig. 2. In vitro effect of (a) zinc and (b) copper on cytotoxicity was tested. Three metal concentrations were used (■) Control, (▩) 10 μg ml $^{-1}$, (▨), 50 μg ml $^{-1}$, (□) 100 μg ml $^{-1}$ for zinc; 5, 10, 20 μg for copper. Kidney cells were incubated for 1 h in RPMI-1640 prior to their addition to the chromium^{51} labelled target cells. The NCC activity of treated kidney cells was measured as previously described. Representative data of 30 fish pooled in each experiment (triplicate). Significant differences with respect to values for controls at $*P<0{\cdot}05$ and $**P<0{\cdot}01$, respectively (Student's *t*-test).

compared to the control group. Effector cells were then tested for NCC activity using a 6 h killing assay against P815 target cells. The pretreatment of the kidney cells to heavy metals was found to affect NCC activity responses (Fig. 2). In comparison to control values, zinc- (50 and 100 μg ml $^{-1}$) and copper-treated (10 and 20 μg ml $^{-1}$) fish kidney cells demonstrated a significant reduction of NCC activity only at the 1:100 ratio. At different E:T ratios, lower zinc and copper concentrations (10 and 5 μg ml $^{-1}$ respectively) produced no change in the cytotoxicity of effector cells as compared to the control group.

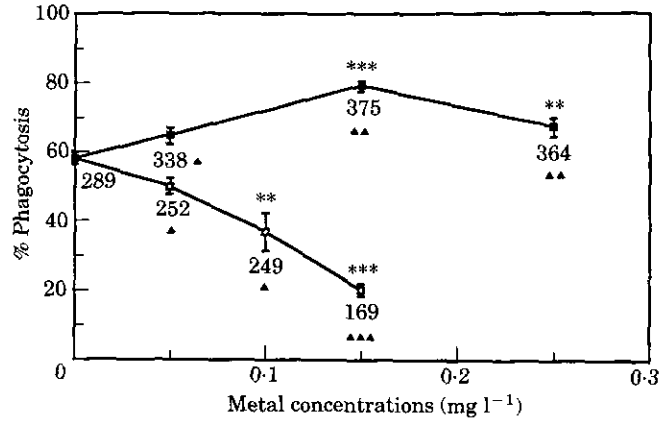

Fig. 3. After 7 days at 22° C, kidney macrophages from both control and metal-exposed fish were tested for phagocytic activity against heat killed bacteria (*A. hydrophila*). (■) Zinc, (□) copper. Phagocyte cells were mixed with bacteria at 1:100 ratio for 180 min at 22° C and the number of phagocytic cells containing bacteria, as well as the number of bacteria per phagocyte were counted. Each data point is the mean ± S.E.M. of 90 fish. Numerals next to the data points indicate the number of bacteria phagocytosed by 100 macrophages. Levels of significance for differences of phagocytosis between the mean of the groups are designated $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$. Significant differences for number of bacteria phagocytosed with respect to values for controls at $\blacktriangle P<0{\cdot}05$, $\blacktriangle\blacktriangle P<0{\cdot}01$ and $\blacktriangle\blacktriangle\blacktriangle P<0{\cdot}001$.

IN VIVO AND *IN VITRO* EFFECTS OF HEAVY METALS ON PHAGOCYTOSIS

The percentage of plastic-adherent mononuclear cells which phagocytosed *A. hydrophila* and the number of *A. hydrophila* within 100 mononuclear kidney cells were significantly altered by both *in vivo* and *in vitro* exposure to zinc and copper (Figs 3 and 4). Phagocytic activity increased with increase in incubation time, reaching maximum values within 180 min for both control and metal-exposed fish. The phagocytic activity of the kidney macrophages was significantly increased in the zinc-exposed group at 0·15 and 0·25 mg l^{-1} of zinc, but at 0·05 mg l^{-1} phagocytic activity was not altered, as compared to that of the control group.

The phagocytic activity in copper-exposed fish was significantly decreased at the 0·10 and 0·15 mg l^{-1} concentrations. At 0·05 mg l^{-1} of copper, no significant change was observed (Fig. 3).

The *in vitro* effects of zinc and copper were also found to alter phagocytic activity (Fig. 4). Phagocytic activity increased significantly in all three zinc-treated concentrations as compared to the control group. In contrast, phagocytic activity decreased significantly in 10 and 20 μg ml^{-1} copper-treated cells. No significant difference was observed in kidney macrophages treated with 5 μg ml^{-1} of copper compared to the control group.

IV. Discussion

Experimental exposure of *B. rerio* to copper and zinc during a 7-day period was found to affect lymphoid cell numbers, spontaneous natural cytotoxicity

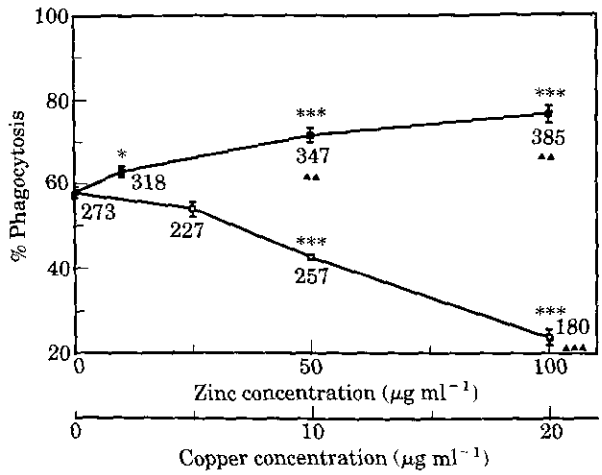

Fig. 4. In vitro effect of (□) copper and (■) zinc on phagocytic activity of *B. rerio* kidney macrophages. Before the phagocytic assay, adherent-cells were treated with different metal concentrations for 1 h at 28° C. The percent phagocytosis was determined as for Fig. 3. Each data point is the mean ± S.E.M. of 90 fish. Numerals next to the data points indicate the number of bacteria phagocytosed by 100 macrophages. Levels of significance for differences of phagocytosis between the mean of the groups are designated $*P<0{\cdot}01$ and $***P<0{\cdot}001$. Significant differences for number of bacteria phagocytosed with respect to values for controls at ▲▲$P<0{\cdot}01$ and ▲▲▲$P<0{\cdot}001$.

and macrophage activity. Toxicological manifestations in the immune system, following xenobiotic exposure in experimental animals, may appear as changes in lymphoid organ weight and/or histology and quantitative or qualitative changes in the cellularity of lymphoid tissue or in the numbers of peripheral leucocytes (Descotes, 1986). The lymphoid organs of fish, the spleen and kidney, have been found to be a target for heavy metals: zinc (Chipman *et al.*, 1958) and copper (Baker, 1969). Our results indicate that 7 days of copper exposure caused a dose-dependant decrease in the cellularity of *B. rerio* kidney (Table 1). Similar results have been obtained in other fish species (Khangarot & Tripathi, 1991) and it is known that sublethal levels of copper had a marked influence on haematological parameters (O'Neill, 1981). During zinc exposure, only a low zinc concentration (0·05 mg l^{-1}) caused a significant decrease in the number of lymphoid cells after 7 days of exposure (Table 1). In a human study, treatment with zinc sulphate for 1 month in patients over 70 years of age, was found to increase lymphocyte numbers (Duchateau *et al.*, 1981). Analysis of copper in different organs of feral fish support the hypothesis that the liver is the most important organ for the accumulation of copper in fish (Dallinger & Kantzky, 1985; Wahbeh & Mahasneh, 1987). Unlike copper, zinc is primarily distributed to the skin, muscle and bone and only small amounts are accumulated in the liver and kidney (Pentreath, 1973, 1976; Wicklund, 1990). Nevertheless, from the limited evidence available, it seems that zinc and copper ions interfere with the replication of fish kidney immunocompetent cells. In the present study, the effect of heavy metals on the proliferation of immunocompetent cells in *B. rerio* was not examined.

However, in our laboratory, *in vitro* studies on lymphocytes from carp pronephros have shown that Zn^{2+} has a mitogenic effect, although it acts by a different mechanism from that of PHA or Con A (Ghanmi *et al.*, 1989) and does not affect interleukin-2 production (Ghanmi, 1989). However, at high concentration, Zn^{2+} has been reported to increase the mitogenic response of pronephric lymphocytes from carp, but at low concentrations, it was shown to inhibit lymphocyte proliferation completely (Ghanmi *et al.*, 1989).

In this study, the results demonstrate that the *B. rerio* kidney contains a potent effector population of cytotoxic cells (Table 2) and environmental chemicals such as heavy metals may alter natural cytotoxicity in the exposed fish. Thus, fish exposed to different zinc and copper concentrations showed dose-dependent decreases in NCC cytotoxicity (Fig. 1). In addition, *in vitro* preincubation of NCC in media containing heavy metals was examined. One hour preincubation in different zinc and copper concentrations produced significant dose-dependent decreases in cytolysis (Fig. 2). Further, the inhibitory response was more than five-fold higher when NCC were treated with copper. These data demonstrate that zinc and copper ions, ubiquitous aquatic pollutants, suppress NCC activity in fish. Moreover, copper seems more toxic than zinc for the lytic mechanisms of fish NCC. Several studies suggest a close similarity in lytic mechanisms between human and fish NCC or natural killer (NK) cytotoxicity (Evans *et al.*, 1984*b*; Graves *et al.*, 1984). In mammals, spontaneous natural cytotoxicity is an important cytolytic effector mechanism in resistance to tumours (Warner & Dennert, 1982) and viral diseases (Bukowski *et al.*, 1983). Hetrick *et al.* (1979) exposed rainbow trout, *Salmo gairdneri* Richardson, to haematopoietic necrosis virus following sublethal exposure to copper and demonstrated a significant increase in susceptibility to infection. In analogy with these findings, it is possible that the suppressed resistance to viral infection following copper exposure was caused by a decrease in spontaneous natural cytotoxicity. Ferry and Donner (1984) have reported that zinc induces a decrease of murine natural killer cytotoxicity and our studies confirm this result in fish.

Macrophages are an important part of the cellular immune system, protecting the host by phagocytosing foreign material. Since the phagocytic ability of fish macrophages is an indicator of macrophage function, it is of interest to ascertain the effect of an environmental pollutant on the normal responses of macrophages (Weeks *et al.*, 1986). In the present study, we have shown that the phagocytic activity of kidney macrophages from copper exposed fish was markedly reduced compared to controls in clean water (Fig. 3). We have also reported that *in vitro* macrophage treatment with copper decreases the phagocytic response (Fig. 4). Khangarot & Tripathi (1991) studied the effect of copper exposure on catfish immune functions. They reported that exposure of 0·056, 01·10 and 0·32 mg l^{-1} of copper for 8 days affects the phagocytic response. Unlike copper, our data demonstrated that zinc caused an increase in macrophage activity in both *in vivo* and *in vitro* exposure (Figs 3 and 4). Copper has been linked to outbreaks of infectious disease in fish through *in vivo* laboratory assays. There is mounting evidence that chronic sublethal level of toxicants increases the susceptibility of fish to disease. These results tend to support our earlier findings that sublethal levels of copper induced an

inhibition of resistance to *Listeria monocytogenes* in *B. rerio* and that zinc induced a stimulation of this resistance (Rougier *et al.*, 1992).

Copper toxicity for fish is well documented (Hetrick *et al.*, 1979) and both suppressive and stimulatory effects of zinc have been reported in studies of humoral antibody response (O'Neill, 1981) and cell-mediated immunity (Ghanmi *et al.*, 1989). Our results demonstrate distinct suppression of the NCC and phagocytic response in fish exposed either *in vivo* or *in vitro* to copper. More importantly, zinc can enhance the phagocytic response and decrease NCC activity in fish exposed *in vivo* as well as *in vitro*. These findings support the concept that immunocompetence may be a measure of the biological effects of environmental pollution and that *in vitro* exposure can be used to provide information about *in vivo* toxicities and immunomodulation by pollutants in fish. Nevertheless, a variety of physiological and environmental factors have been shown to modify immune responsiveness and must be noted when conducting immunotoxicological investigations. For example the interrelationship between hormones and the immune system is very important (Faisal *et al.*, 1989; Ndoye *et al.*, 1991, 1992). Moreover, physiological stress has been shown to be associated with alteration of the immune response in fish (Saad, 1988; Peters *et al.*, 1991). Consequently, the interpretation of immune alterations observed during toxicity studies are best based on the results of several immunocompetence assays: lymphoid organ pathology, humoral and cell-mediated immunity, macrophage function, non-specific cytotoxic cell activity and host resistance to infection.

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