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Effects of *In Vitro* Nickel Exposure on the Macrophage-Mediated Immune Functions of Rainbow Trout (*Oncorhynchus mykiss*)

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Nickel is an element which occurs naturally in the geophysical environment. It has been utilized extensively in manufacturing and, hence, has become a common byproduct of industrialization. Nickel is released into the atmosphere by oil and coal-burning power plants and trash incinerators, and is also discharged into waste water by industries which convert scrap or new nickel into alloys (USDHHS 1991). The effluent that spreads to streams, rivers, and lakes may disrupt the integrity of the aquatic environment. Excess nickel contamination is hazardous to aquatic ecosystems due to, among other things, its persistence and bioaccumulation (Atchison et al. 1987). While the adverse health effects associated with nickel exposure have been extensively examined in mammalian systems, very little is known concerning its effects on aquatic organisms.

Although trace amounts of nickel are necessary for maintaining the metabolic homeostasis of some vertebrate species (USDHHS 1991), larger amounts of nickel have been shown to be toxic. In addition to being both genotoxic and carcinogenic (Costa and Heck 1982), nickel modulates immunological functions in a variety of mammalian species (Sunderman et al. 1989; Haley et al. 1990). The toxic effects of nickel on the numbers, activity, and ultrastructure of macrophages (Mø), a crucial cell type involved in cell-mediated immunity, have been well-studied (Bingham et al. 1972; Camner et al. 1978). While similar information regarding the effects of nickel on aquatic organisms is lacking, it has been reported that a number of toxic metals such as copper, manganese, and cadmium modulate the immune responses of fish (Reviewed in Zelikoff 1993). Consequently, examining the immunotoxicity of nickel to fish may be of special importance. To appraise the immunomodulating potential of nickel on fish, and to begin to establish baseline parameters of altered immune function as potential biomarkers of *in vivo* nickel exposure, elicited peritoneal macrophages from rainbow trout (*Oncorhynchus mykiss*) were treated *in vitro* with increasing concentrations of nickel sulfate ($NiSO_4$). Following exposure, Mø activities important for maintaining host immunocompetence were evaluated and these include; mobility (random and stimulus-directed), production of reactive oxygen intermediates (ROI), acid phosphatase activity, and phagocytosis.

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MATERIALS AND METHODS

Rainbow trout (*Oncorhynchus mykiss*), weighing 350-500 g, were purchased from Muskey Trout Hatchery (Asbury, NJ). The fish were maintained in well-aerated aquaria at 10°C ± 4°. Trout chow was given daily and any unconsumed food was removed within five hours after feeding. Trout were acclimated in the laboratory for at least ten days before use.

Peritoneal Mø were elicited by two intraperitoneal injections of 0.4 mg *Aeromonas salmonicida* in Freund's incomplete adjuvant, fourteen and seven days prior to sacrifice. Macrophages were collected by lavage after anaesthetization with MS-222 (3-amino benzoic acid ethyl ester, pH 7.0). The lavage fluid was centrifuged at 400x g and the cell pellet resuspended in Eagle's Minimal Essential Medium with Earle's salts (EMEM-E). Cell aliquots were taken to assess Mø number and viability by hemocytometer counting and trypan blue exclusion, respectively, and the remaining cells were used for the functional assays.

The Mø used to assess migration were resuspended in Gey's Balanced Salt Solution (GBSS) supplemented with 2% bovine serum albumin. After a 90 min incubation with $NiSO_4$ at concentrations ranging from 10 to 1000 µM, cells were loaded into the upper well of blind-well microchambers separated by Millipore filters (5 µm pore-size) (Neuroprobe, Cabin John, MD). Zymosan-activated trout serum (C5a) at a concentration of 0.05% (in GBSS) served as the chemoattractant for assessing stimulus-directed movement. Migration was quantitated after 3 hr (at 16°C) by counting the number of Mø migrating to the leading front of the filter per 20 oil immersion fields (Zelikoff et al. 1991).

Measurement of superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) production was based on a method originally described by Pick (1986) and modified for trout Mø by Zelikoff et al. (1991). Measurement of $O_2\cdot^-$ was dependent upon the superoxide reduction of ferricytochrome c; H_2O_2 production was determined by the H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red. Macrophages used to determine the effects of $NiSO_4$ on the production of ROIs (i.e., H_2O_2 and $O_2\cdot^-$), were added to 96-well microtiter plates at 2 x 10^5 cells/well. After a 2 hr attachment period, Mø were washed (reserving the supernatant to determine the number of detached cells), and either 100 or 250 µM $NiSO_4$ was added. Cells were then reincubated at 16°C for an additional 90 min with and without phorbol myristate acetate (PMA), a stimulator of the respiratory burst in fish and mammals (Secombes et al. 1988; Frenkel 1989; Zelikoff et al. 1991). Production of H_2O_2 and $O_2\cdot^-$ was measured spectrophotometrically at 550 nm and 600 nm, respectively. The results are expressed as nmol ROIs/2 x 10^5 cells/60 min.

To assess total acid phosphatase activity, naive Mø (5 x 10^5/well) were incubated (90 min at 16°C) in double-welled chamber slides with $NiSO_4$ at concentrations ranging from 10 to 1000 µM. Levels of total acid phosphatase (both intra- and extracellular) were measured

spectrophotometrically at 420 nm using commercially available kits (Sigma, St. Louis, MO).

Following incubation with $NiSO_4$ for 90 min (at 16°C), phagocytosis of fetal calf serum (FCS)- opsonized latex particles (< 3 μm diameter; Duke Scientific, Palo Alto, CA) by trout Mø (2 x 10^5/well) was determined using a monolayer assay system as described by Enane (1991). Phagocytic activity was expressed as the phagocytic index (percentage of Mø containing at least one particle) and phagocytic capacity (percentage of cells engulfing a specific number of particles). Results were based upon oil-immersion examination of 200 cells/Ni dose.

The results of the immunological assays for the control and nickel-treated cells were compared using a Student's t-test. Statistical significance was considered at $p<.05$.

RESULTS AND DISCUSSION

Our findings indicate that at concentrations as high as 1 mM, exposure of trout peritoneal Mø to $NiSO_4$ does not: a) alter intracellular production or extracellular release of acid phosphatase; b) reduce the viability of rainbow trout elicited peritoneal Mø (as determined by trypan blue exclusion) nor; c) alter the phagocytosis of opsonized latex particles as compared to values observed for nickel-free control cells. These data are consistent with *in vivo* and *in vitro* studies which demonstrated that exposure of mammalian Mø to nickel compounds does not significantly decrease cell viability nor affect phagocytic activity, except following long-term (> 20 hr) exposure to nickel at concentrations in excess of 1 mM (Waters and Gardner 1975; Tam and Hinsdill 1984; Sunderman et al. 1989). It is possible that nickel may also produce effects on trout Mø similar to those observed for mammalian cells at higher nickel concentrations and/or after longer exposure durations.

In contrast to the lack of effects noted above, exposure to $NiSO_4$ at 100 and 250 μM produced a 3-fold statistically significant ($p<0.05$) depression in spontaneous H_2O_2 production by trout peritoneal Mø (Figure 1) as well as a 30% reduction in basal levels of $O_2\cdot^-$ after exposure to 100 μM nickel (Figure 2). Since nickel has been shown to inhibit the enzyme responsible for the dismutation of $O_2\cdot^-$ to H_2O_2 (Shainkin-Kestenbaum et al. 1991), the observed reduction in H_2O_2 production could have been due to nickel-induced alterations in superoxide dismutase (SOD). If this were the case, however, one might expect a concurrent increase in the accumulation of $O_2\cdot^-$ (due to a lack of dismutation), which was not observed. This suggests that nickel interferes at an earlier step of ROI production. Since exposure to nickel has been shown to change Mø ultrastructure and cause damage to the cell membrane (Camner 1978; Johansson et al. 1983; Sunderman et al. 1989), it is possible that nickel causes a depression in H_2O_2 and $O_2\cdot^-$ production by interfering with the membrane-bound oxidases or with cytochrome b, important in the initial stages of molecular oxygen consumption and the ultimate conversion to H_2O_2 (Fridovich 1978; Frenkel 1989).

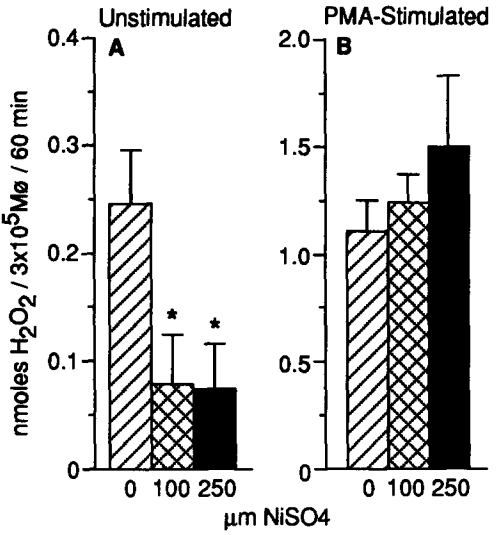

Figure 1. Production of H_2O_2 by unstimulated (A) and PMA-stimulated (B) trout peritoneal Mø exposed *in vitro* to 100 and 250 µM $NiSO_4$ for 90 min. H_2O_2 production by unstimulated Mø was significantly (*$p<.05$) depressed by exposure to $NiSO_4$. Values represent the means ± SE of 9 fish. Note the difference in scale between (A) and (B).

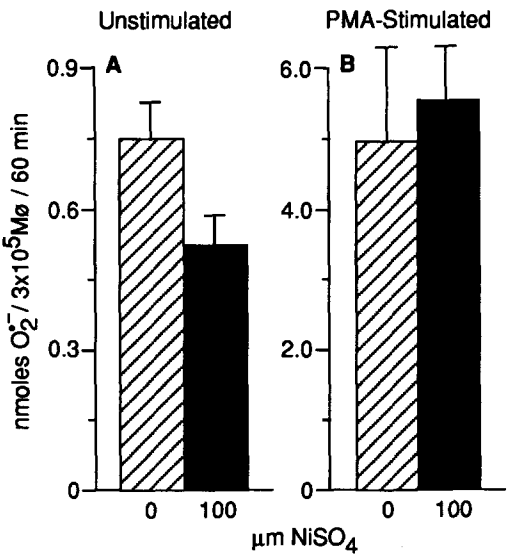

Figure 2. Production of $O_2^{\bullet-}$ by unstimulated (A) and PMA-stimulated (B) trout peritoneal Mø exposed to 100 µM $NiSO_4$. Values represent the means ± SE of 4 fish. Note the difference in scale between (A) and (B).

Elicited trout peritoneal Mø produce ROIs as a microbicidal defense mechanism, and their production may be enhanced by *in vitro* stimulation with PMA (Zelikoff et al. 1991). Trout Mø exposed to $NiSO_4$ and then stimulated *in vitro* with PMA, produced increased amounts (over that of unexposed stimulated controls) of both $O_2^{\cdot -}$ and H_2O_2 (Figures 1B and 2B). Stimulation of $O_2^{\cdot -}$ and H_2O_2 production by the addition of PMA appears to mask the inhibitory effects on spontaneous ROI formation produced by nickel (Figures 1A and 2A). The possible effects of nickel on the interactions comprising this response requires further investigation to clarify the underlying mechanisms involved.

Random migration of trout peritoneal Mø was significantly elevated (3-5 fold) by exposure to 10 and 100 µM nickel for 90 min; stimulus (C5a)-directed movement was also enhanced but only by 10 µM nickel (Figure 3). At concentrations greater than 10 µM, Mø movement declined with increasing nickel concentrations reaching control levels by 500 µM. In addition, the magnitude of the response to the chemoattractant was reduced by exposure to increasing nickel concentrations. Migration of control Mø (no nickel) was significantly

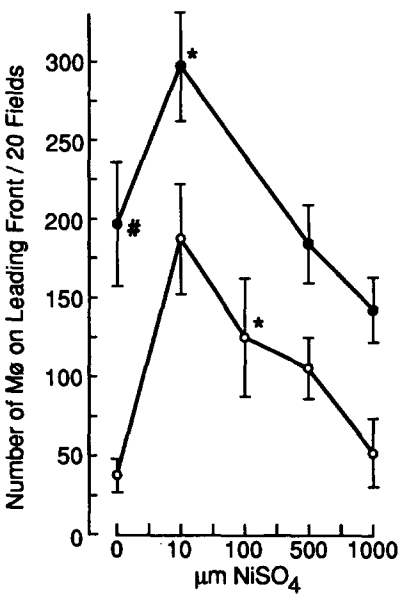

Figure 3. Total number of control (no nickel) and $NiSO_4$-exposed trout peritoneal Mø (per 20 oil-immersion fields) moving randomly (o) and in response to the chemoattractant C5a (o). Random migration and C5a-directed movement was significantly increased (*$p<.05$) by exposure to nickel. A significant difference (#$p<.05$) was also noted between random and stimulus-directed movement by control Mø. Values represent the means ± SE of 5 fish.

enhanced (5-fold above random migration) by their exposure to C5a; exposure to $NiSO_4$ reduced Mø response to C5a stimulation by more than 50%. The ability of Mø to migrate randomly and towards a stimulus is essential for immune surveillance by the host (Warheit et al. 1986), thus, any change of this vital function can influence host resistance to infectious agents and to the growth of tumors. The ability of trout peritoneal Mø to respond chemotactically to C5a suggests the presence of surface receptors similar to those reported for guinea pig alveolar Mø. The formation of protrusions, microvilli and laminated structures on the Mø surface has been associated with increased Mø activation (Zelikoff and Enane 1991), and thus, enhanced cell motility. Since nickel has been shown to increase the number of membrane surface projections on rabbit alveolar Mø (Camner et al. 1978), it is possible that migration of nickel-exposed trout Mø was enhanced because of its effects on the activation state of those cells.

In summary, this study showed that exposure of elicited trout peritoneal Mø to $NiSO_4$ at nickel concentrations somewhat greater than those found in the polluted aquatic environment (0.2 μM; USDHHS) and below those shown to substantially reduce Mø viability, altered Mø functions important for maintaining host resistance to infectious agents and tumor growth (i.e., Mø migration and basal production of ROIs). While soluble nickel did not appear to concentrate in fish (USDHHS), this study suggests that exposure of feral fish to relatively low concentrations of nickel such as those found in polluted aquatic environments or following "accidental spills," may alter immunoregulatory mechanisms important for maintaining host immunocompetence. In addition, results from this *in vitro* study support the laboratory and field data (Reviewed by Zelikoff 1993) which suggest a causal relationship among aquatic pollution, infectious diseases, and developing neoplasms in feral fish populations, *via* effects on the immune system.

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