Multicellular organisms employ a finely balanced array of immunological defenses to prevent infection with naturally occurring opportunistic pathogens in their environment. Feedback mechanisms regulate the magnitude of the immune response while cell surface recognition molecules assist in the selection of appropriate non-self antigen targets to avoid damage that might arise from autoimmune reactions. Although its specific mechanism of action remains unclear, environmental mercury exerts a biphasic, dose-dependent effect on the mammalian immune system. High doses of organic and inorganic mercury can suppress responses, as measured by in vitro and in vivo assays of immune function (1–3). In contrast, subtoxic doses of mercury may activate the immune system. The consequences of this inappropriate activation include autoimmune pathologies such as contact dermatitis, immune glomerulitis, and hypergammaglobulinemia (4), suggesting that activating doses of mercury may interfere with regulatory processes that control the magnitude and specificity of immune effector cell function.

High doses of mercury are also toxic to the immune defense systems of many lower vertebrates, including chicken and fish (5–7). It has been suggested that low doses of mercury might also activate the immune systems of nonmammalian species (7,8), although direct demonstration of this phenomenon has not been provided. Teleost fish such as Sciaenops ocellatus, the red drum, possess neutrophils, macrophages, T-cell subsets, and B lymphocytes that are comparable to those of mammals (9). The present study explored the possibility that HgCl₂ might have a biphasic, dose-dependent effect on cells of the red drum immune system. Responses to in vitro mercury exposure were evaluated by monitoring DNA synthesis as well as tyrosine phosphorylation and calcium flux, which are critical early events in cellular activation.

Materials and Methods

Cell Culture and Proliferative Assays

Red drum (1–2 years old; 1–2 kg) were obtained from the aquaculture program of the Marine Resources Research Institute of the South Carolina Department of Natural Resources (Fort Johnson, SC). Animals were maintained at 25 ± 2°C in sea water at 20 ppt NaCl in a recirculating drip-filtration system. Animals were fed squid and shrimp daily. All animals were acclimated to the holding tanks for a minimum of 30 days prior to blood sampling.

Peripheral blood leukocytes (PBLs) were isolated by Ficoll gradient centrifugation (10). Unless otherwise indicated, red drum cell cultures were at 2.5 × 10⁸ cells/ml at 25°C in a standard tissue culture medium RPMI 1640 made isotonic with red drum plasma (approximately 360 mosmol/kg) by adding 0.21 g NaCl/100 ml medium (11). Osmotically adjusted RPMI 1640 (RDRPMI) was supplemented with 5% red drum serum and 5% human serum, as previously described (11), to optimize PBL viability and growth. A catfish B cell line, 1B10, kindly provided by N. Miller, University of Mississippi Medical Center, was maintained and treated in culture medium supplemented with 2% catfish serum (12). The human lymphoblastoid cell line MP-8 was obtained from J. M. Gouat, The Medical University of South Carolina (13).

Stock solutions of the mitogens lipopolysaccharide (LPS, 1.0 mg/ml) and Concanavalin A (ConA, 0.5 mg/ml) were prepared in RPMI 1640, aliquoted, and stored at -70°C. Stock solutions of 100 mM HgCl₂ and 100 mM diethiothreitol (DTT) were prepared in double distilled water and stored at 5°C. Phorbol ester 13-O-tetradecanoylphorbol acetate (TPA) was prepared in dimethyl sulfoxide (DMSO) as a 0.33 M stock solution, aliquoted, and stored in light-tight containers at -70°C. Immediately before use, these stock reagents were diluted in culture medium to the appropriate working concentrations. Stocks prepared in DMSO were diluted to a minimum of 1:1000 to minimize background effects. The mitogens DMSO, DTT, and HgCl₂ were from Sigma Chemical Company (St. Louis, MO).

For proliferation assays, cells were plated in 96-well microculture plates at 4.5 × 10⁵ cells/well in 180 μl of the specified medium; 20-μl amounts of the diluted mitogen or immunomodulator were added, as appropriate. All mitogens and immunomodulators were removed from the wells after 18 hr of treatment, with minimal disturbance to the cells, and replaced with medium alone. Unless otherwise indicated, red drum PBLs were cultured for an additional 30 hr, while catfish B cell line 1B10 and human lymphoblastoid cell line MP-8 were cultured for 6 more hours prior to measuring DNA synthesis. At the end of the indicated culture times, 0.5 μCi ³H-thymidine (NEN Research Products, Boston, MA) was added per well, and cells were harvested 18 hr later on glass fiber filters (Cambridge Technology, Watertown, MA). Uptake of radiolabeled thymidine was assessed using a liquid scintillation counter. The indicated times, cell proliferation rates were quantified as the ratio of radiolabeled ³H-thymidine over 18 hr. Each illustrated data point represents the average of triplicate values ± SE. Significance was calculated using the Students t-test. A regression line was fitted to the linear portion of the inhibition curve.
from each experiment and used to calculate the IC$_{50}$ (concentration that inhibits 50%).

To compare the time required for HgCl$_2$ to activate or inhibit cell proliferation, freshly isolated red drum PBLs were treated in parallel in one of three ways. For short-term exposures, cells were treated in batch culture for 10 min with 2 ng/ml TPA and 1 mM HgCl$_2$, washed by centrifugation, and returned to culture in individual microtiter plate wells with TPA alone for 18 hr. For long-term exposures, PBLs were treated continuously for 18 hr with 2 ng/ml TPA and 1 mM HgCl$_2$ in individual microtiter plate wells. To control for possible detrimental effects of the short-term exposure washing procedure, cells from the same fish were treated in batch culture for 10 min with 2 ng/ml TPA and 1 mM HgCl$_2$, washed by centrifugation, and placed in microwell cultures with the same doses of TPA and HgCl$_2$ for 18 hr. After the indicated exposure periods, all treatments were removed from the wells with minimal disruption to the cell layer. New media without mitogens was added for an additional 30 hr, after which cell proliferation was measured as the uptake of $^3$H-thymidine over 18 hr and calculated as indicated above.

Western Blots for Tyrosine Phosphorylation

Red drum PBLs were resuspended at 5 x 10$^6$/ml, treated with 0, 1, 10, or 100 mM HgCl$_2$ for 1 or 5 min, and centrifuged at 1200 rpm in a microcentrifuge for 5 min. The resulting pellet was resuspended in lysis buffer [20 mM Tris-HCl, pH 7.5; 0.1% Triton X-100; 1 mM EGTA (ethyleneglycoltetraacetic acid); 2 mM EDTA; 5 mM aprotinin; 5 mM phenylmethylsulfonyl fluoride] at 1 $\mu$L/10$^6$ cells. The cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions along with molecular weight standards. The resulting gels were stained for protein with Coomassie blue and probed by standard Western blot techniques (14) using antiphosphotyrosine antibody PY20 (Transduction Laboratories, Lexington, KY). Antibody that bound to the Western blot was detected using a goat anti-mouse antibody conjugated with horseradish peroxidase. The bound antibodies were imaged by enhanced chemiluminescence (ECL: Amersham Life Sciences, Arlington Heights, IL). The sizes of phosphorylated proteins were calculated from direct measurement of their migration distances on the gel against a linear regression curve generated from standards included on the same gel. Measurements were confirmed on a second gel by a separate observer.

Calcium Assays

Calcium flux was measured using freshly isolated PBLs washed and resuspended in RDRPMI at 5.0 x 10$^5$/ml TPA, HgCl$_2$, or DMSO control were added, followed by 0.5 $\mu$Ci/ml $^{45}$Ca$^{2+}$ (NEN Research Products). Cells were incubated with radiolabel at room temperature for 10 min, placed on ice, and washed twice in cold phosphate buffered saline (PBS). The final cell pellet was resuspended in 100 $\mu$l lysis buffer (1% SDS in 0.5 N NaOH), dissolved in Ecoscint O (National Diagnostics, Atlanta, GA) scintillation cocktail, and quantified using a liquid scintillation counter. Each illustrated data point represents the average of duplicate assays ± SEM with significance calculated using the Students t-test.

To assay intracellular calcium levels, freshly isolated PBLs were loaded with 2.5 $\mu$M fura-2 (Sigma) for 30 min. Cells were washed by centrifugation and resuspended in Dulbecco’s PBS at 10 x 10$^6$ cells/µl. All HgCl$_2$ and DTT concentrations were added as 10 µl aliquots directly to 1 ml of cells in quartz cuvettes. Relative calcium concentration was monitored by fluorescence absorbance using an SLM 800 spectrophotometer (SLM Aminco, Rochester, NY). The monochromator was set at 360 nm for excitation and 510 nm for emission. Absorbance changes were monitored over 1000 sec. Where appropriate, MnCl$_2$ (40 µM) was added at the termination of experiments to assure that extracellular fura-2 did not contribute substantially to the observed fluorescence signal.

Results

Concentration and Time-dependent Effects of HgCl$_2$ on PBL Proliferation

In the absence of exogenous mitogens to stimulate cell growth, cultures of red drum PBLs did not proliferate substantially (100–300 cpm $^3$H-thymidine). HgCl$_2$ had no statistically significant effect on these unstimulated cultures (data not shown). When red drum PBLs were stimulated with optimal mitogenic doses of TPA (20 ng/µl) in the presence of various concentrations of HgCl$_2$, the proliferative response was completely inhibited by concentrations of mercury $\geq$10 µM (Fig. 1). A long-term B cell line derived from channel catfish, B110 (12), and a human lymphoblastoid cell line, MP-8 (12), were cultured with the same concentration range of HgCl$_2$. The concentrations that produced 50% inhibition of maximum proliferation (IC$_{50}$) were similar for the primary red drum PBLs, teleost B cell line, and human lymphoblastoid cell line, within the range of 1.0–10.0 µM HgCl$_2$ (Fig. 1). The calculated IC$_{50}$ was 4.8 µM HgCl$_2$ (95% confidence limits 3.4, 6.2 µM) over 10 replicate studies with red drum PBLs. PBL activated with optimal or suboptimal levels of the B cell mitogen LPS or T cell mitogen ConA also were inhibited by HgCl$_2$ with 1.0 < IC$_{50}$ < 10.0 µM in two animals tested (Fig. 2).

In contrast, when red drum PBLs were stimulated with submitogenic concentrations of TPA, HgCl$_2$ dramatically enhanced cell proliferation, as measured by the uptake of $^3$H-thymidine into DNA (Fig. 3). The action of TPA and HgCl$_2$ on the proliferative response was synergistic because their combined effects exceeded the expected additive effect of each reagent alone. In PBLs from all three animals tested, 2 ng/ml TPA achieved maximum synergism with 0.1–1.0 µM HgCl$_2$ causing the proliferative responses to increase by 2.6, 2.7, and 5.9-fold over 2 ng/ml TPA alone (p<0.001 for each experiment). Low concentrations of HgCl$_2$ did not routinely enhance proliferation in cells treated simultaneously with suboptimal mitogenic levels of LPS or ConA, although small, insignificant increases were often observed (Fig. 2).

To determine whether it might be possible to distinguish the cellular targets for HgCl$_2$-mediated toxicity and enhancement, we tested whether these cellular responses were differentially effected by the length of exposure to mercury. Freshly isolated PBLs were incubated for 10 min with TPA and HgCl$_2$, then washed and cultured in TPA alone or TPA + HgCl$_2$. The results of this experiment are shown in Figure 4.

![Figure 1. Inhibition of proliferation in human MP-8 cells, catfish B110 cells and red drum peripheral blood leukocytes (PBL) by HgCl$_2$. Results provided for PBL are from a single animal, representative of more than 10 animals tested. Error bars indicate SE.](image-url)
As observed in previous experiments (Fig. 1, 3) and in all three animals tested, an 18-hr exposure to high concentrations of HgCl$_2$ (≥10 μM) completely suppressed PBL proliferation induced by 2 ng/ml TPA. Lower concentrations of mercury (0.1–1.0 μM) synergized with TPA to enhance cell growth significantly (1.9, 2.7, and 4.9-fold, with p<0.001 for each experiment). A 10-min exposure to 10 μM HgCl$_2$ also suppressed PBL proliferation induced by the phorbol ester. However, a 10-min treatment with 0.1–1.0 μM HgCl$_2$ was not sufficient to induce a significant enhancement of cell growth in any of the three animals tested. These results suggest that low concentrations of mercury might be inducing cellular responses which could be distinguished from toxic effects.

**Induction of Tyrosine Phosphorylation by HgCl$_2$**

To test whether mercury might activate cells by inducing rapid tyrosine phosphorylation of cellular proteins, freshly isolated red drum PBLs were exposed to 1, 10, or 100 μM HgCl$_2$ for 1 or 5 min. Treated cells were lysed and their proteins were separated by SDS-PAGE and stained by Western blot with anti-phosphotyrosine antibody PY20 (Fig. 5). Treatment with 10 μM HgCl$_2$ stimulated phosphorylation of tyrosine residues on numerous proteins, which were initially detected within 1 min. Discrete phosphotyrosine proteins appeared at approximately 41, 46, 56, and 60 kDa, with four additional prominent bands at 100–200 kDa. The extent of phosphorylation on the four lower molecular weight proteins declined at 5 min of treatment, particularly in the 60 kDa band. Treatment with 100 μM HgCl$_2$ rapidly induced tyrosine phosphorylation on a large number of additional protein bands in the range of 40–200 kDa. Band intensity was similar after 1 and 5 min of treatment; however, 1 μM HgCl$_2$, a concentration that synergized with phorbol ester to induce cell proliferation, failed to induce detectable tyrosine phosphorylation in cell lysates after either treatment period.

**Induction of Calcium Flux**

To evaluate whether short-term calcium fluxes were induced in PBLs by treatment with mercury we measured uptake of $^{45}$Ca into freshly isolated PBLs exposed to a wide range of mercury concentrations using calcium ionophore A23187 (CI) as a positive control (Fig. 6). In separate experiments with cells from three animals, HgCl$_2$ ≥25 μM induced significant calcium influx (p<0.005). Calcium uptake increased with increasing concentration of HgCl$_2$ (Fig. 6A) and duration of exposure (up to 60 min, Fig. 6B). Although this technique demonstrated that calcium flux was associated with short-term exposure to high concentrations of mercury, no significant movement of calcium could be detected in any PBL cultures exposed to ≤10 μM HgCl$_2$.

To evaluate the possibility that synergistic concentrations of HgCl$_2$ might stimulate slow or low-level calcium flux into PBLs, we monitored intracellular calcium levels using the fluorescent calcium indicator dye, fura-2. PBLs were loaded with fura-2 for 30 min, washed, resuspended in PBS, and loaded into the cuvette of a spectrofluorometer. When treated with 1 μM HgCl$_2$, the cells displayed a slow rise in intracellular calcium, reaching a plateau at approximately 2 min after addition of mercury (Fig. 7).
This elevated calcium level, monitored as an approximate twofold increase in the fluorescence ratio of fura-2, was sustained over the remaining time course of the study, i.e., an additional 10 min. Addition of the reducing agent, 1 mM DTT, blocked the rise in intracellular calcium, suggesting that calcium flux induced by low concentrations of HgCl₂ required the cross-linking activity of divalent Hg²⁺. Tracings similar to those shown in Figure 7 were obtained with PBLs from the four additional animals tested. The results of these experiments with fura-2 showed that concentrations of HgCl₂, which synergistically activated cells of the fish immune system, could induce the kind of low-level, sustained increases in intracellular free calcium that are required to initiate cell division.

Discussion

Natural vertebrate populations, including ecologically and commercially important marine fish like the red drum, rely upon their immunological defenses for protection against bacterial, viral, and fungal pathogens in the environment. The studies reported here have shown that toxic concentrations of HgCl₂ induced massive calcium flux and activation of tyrosine kinase activity in teleost PBLs. By comparison, subtoxic concentrations of inorganic mercury that stimulated cell proliferation also triggered small sustained increases in free intracellular calcium. These studies support the contention that subtoxic doses of HgCl₂ could alter the activation state of the fish immune system, potentially disrupting its critical accuracy and feedback controls.

Studies in rats, mice, and humans indicate that low concentrations of mercury may activate lymphocytes by influencing critical signaling pathways. Several mechanisms have been proposed to explain this activation phenomenon. For example, mercury may target specific enzymes such as protein kinase A or protein kinase C (15). Alternatively, it may subtly alter the overall oxidative status of the cell (16). Recently, Nakashima et al. (17) provided evidence that divalent mercury induced receptor aggregation on cell surfaces, bypassing the normal requirement for specific ligand–receptor interaction. Such a general cross-linking event might trigger inappropriate autophosphorylation and activation of numerous receptor-associated tyrosine kinases, with a cumulative effect of inducing cell proliferation. In our study, high concentrations of HgCl₂ (10 and 100 µM) induced a distinctive and transient pattern of tyrosine phosphorylation on red drum PBL proteins, including the appearance of 56 and 60 kDa phosphotyrosine proteins. The latter two proteins were of similar size to phosphorylated proto-oncogene proteins previously identified in receptor-activated nonspecific cytotoxic cells of the channel catfish, Ictalurus punctatus (18). In contrast, a lower 1 µM dose of HgCl₂, which stimulated proliferation in TPA-treated cells, did not induce detectable levels of tyrosine phosphorylation.

Small sustained increases in cytosolic free calcium levels also might play an important role in leukocyte activation. Excessive elevations can activate a large number of proteases, phospholipases, and

Figure 6. Uptake of ⁴⁵Ca induced by HgCl₂. (A) Dose titration; treatment with calcium ionophore A23187 (CI) served as a positive control. (B) Calcium uptake in peripheral blood leukocytes treated for various times up to 60 min with 10 or 25 µM HgCl₂. *Indicates treatments producing significant calcium influx (p < 0.005); results provided are from a single animal representative of three animals tested.

Figure 7. Calcium flux in fura-2 loaded red drum peripheral blood leukocytes exposed to 1 µM HgCl₂. Where indicated, the thiol-protecting reagent dithiothreitol (DTT; 1 µM) was added simultaneously with HgCl₂, directly to the cuvettes. Results provided are from a single animal.
endonucleases that are toxic to the cell (19–21). Red drum PBls stimulated with 1 μM HgCl₂ displayed a twofold rise in fura-2 fluorescence ratio, which was remarkably similar to the kinetics and duration of calcium flux observed in mam-
malian lymphocytes treated in a similar fashion (22).

Even in unpolluted areas, natural fish populations may have substantial body burdens of mercury (50–400 μg Hg/kg), of which 20–40% is inorganic (23). In areas tainted with mercury, fish bioaccumulate and biomagnify this metal largely through lower trophic feeding. Once consumed, a substantial fraction of inorganic mercury becomes covally bound in tissues (24). The extent to which sufficient free mercury may be available in vivo to alter the activation state of the immune system remains to be determined. The in vivo consequences of these mercury-induced activation events on the fish immune response also must be clarified. Nonetheless, the observation that HgCl₂ can alter activation events in fish leukocytes supports the contention that critical subtoxic doses of environmental mercury may be a serious factor in evaluating the ability of aquatic habitats to support healthy natural fishery populations.

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