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Low-level Mercury Causes Inappropriate Activation in T and B Lymphocytes in the Absence of Antigen Stimulation

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Running Title: Low-level Mercury Induces Lymphocyte Activation

Abstract

The immune system primarily utilizes two cell types for adaptive immunity: T lymphocytes and B lymphocytes. T lymphocytes are activated when antigen presenting cells (APCs) present antigen to membrane-bound T cell receptors. B lymphocytes are activated when an antigen binds to receptors embedded in the plasma membrane. In both T and B cells this antigen binding crosslinks the receptor complexes and initiates the signal transduction cascade. These cascades frequently consist of a series of intracellular molecules becoming phosphorylated in a step-wise fashion. Once activated, these cells differentiate into effector cells that clear out the stimulating antigen. Mercury, which is a widespread environmental contaminant, is known to affect the immune system by increasing the potential for autoimmunity. The mechanisms triggered by low-level mercury exposure that cause an organism's immune system to attack its own tissues are currently unclear. In this study, populations of EL4 T cells and WEHI-231 B cells were exposed to multiple low-level concentrations of HgCl_2 for either 12 or 96 hours to mimic acute and chronic subtoxic exposure. Analytical intracellular flow cytometry was used to detect the presence of fluorescent-labeled antibodies bound to phosphorylated forms of multiple activation molecules in the signal transduction cascade. In the absence of antigenic stimulation, increased levels of activation molecules were present within both types of immune cells following low-level mercury exposure, indicating inappropriate activation of these cells by the mercury alone.

Introduction

Inorganic mercury (Hg) is a widespread environmental toxin. Methanogenic bacteria transform mercuric chloride into organic methylmercury (CH_3HgCl) and dimethylmercury ($\text{CH}_3\text{CH}_2\text{HgCl}$)

(Tchounwou et al. 2003). These organic compounds are prone to entering water supplies and food chains where humans are most likely to ingest them (Zahir et al. 2005). Mercury has well-documented neurotoxic and immunological effects on humans and mice (Rowley and Monestier 2005).

Immunological effects in rats and mice include elevated serum levels of immunoglobulin G1 (IgG1), immunoglobulin E (IgE) and the formation of renal IgG deposits (Hultman and Enestrom 1989). Autoantibody production, particularly antinucleolar autoantibodies, is characteristic of mercury-induced autoimmunity (Chen and von Mikecz 2000, Pollard et al. 1997). This autoimmunity is most often studied in mice that have H-2^s, H-2^b or H-2^d genetic backgrounds because they are predisposed to developing autoimmunity in response to heavy metal exposure (Hultman et al. 1994, Warfvinge et al. 1994, Abedi-Valugerdi and Moller 2000, Hansson and Abedi-Valugerdi 2004, Silva et al. 2005, Laiosa et al. 2007).

It has been shown in animal models that exposure to xenobiotics, including mercury, leads to the development of autoimmune diseases (Via et al. 2003, Silva et al. 2004). In humans, exacerbation of scleroderma, lupus and rheumatoid arthritis, as well as neurological dysfunction, has been documented (Verwilghen et al. 1992, Yokoo et al. 2003, Silbergeld et al. 2005, Copper et al. 2008). Mercury can also affect perinatal development when exposure occurs before birth (Dietert and Piepenbrink 2006). Our study examines longer exposure and subtoxic doses of murine lymphocyte cell lines to mercury, examining alterations in signal transduction molecules following exposure. This exposure level may be more like that which humans encounter in diet and environment.

Previous work in the available body of literature has focused on exposing immune cell lines to high concentrations of HgCl_2 for short durations of time. Several studies found attenuation in activation of WEHI-231 B cells and Jurkat T cells following *in vitro* exposure to mercury. Activation of lymphocytes

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commences with stimulation through their surface-bound antigen receptors, and can be artificially achieved via anti-Ig μ and anti-CD3 antibodies for B and T lymphocytes, respectively. Once the cells are activated, signal transduction cascades commence to push the cells into the cell cycle for proliferation. These signal transduction cascades frequently involve phosphorylation of cytoplasmic and/or cytoplasmic portions of membrane-bound proteins. In vivo, this proliferation would be accompanied by differentiation into effector forms (such as antibody-producing plasma cells and helper/cytotoxic T lymphocytes) to carry out the immune responses. (McCabe et al. 1999, McCabe et al. 2007, Ziemba et al. 2009, Limnander and Weiss 2011). In these studies, WEHI-231 B cells were exposed to 5 μ M HgCl $_2$ for 15 minutes and Jurkat T cells to 5 μ M HgCl $_2$ for 5 minutes. These studies indicated elevated activation in negative control samples when exposed to mercury alone in the absence of stimulation, but did not discuss these results (McCabe et al. 1999, Ziemba et al. 2009).

The mercury concentrations and exposure times of the McCabe and Ziemba studies are of a questionable physiologic relevance. It has been cited that 0.1 μ M mercury is a biologically relevant dose and that serum concentrations in exposed human populations are roughly 0.15 μ M mercury (Druet et al. 1989, Ratcliffe et al. 1996). In a later study by McCabe et al., exposure times were expanded somewhat to 30 minutes. Mercury levels used remained high at 20 μ M and 10 μ M (Gill et al. 2014). This 2014 study also utilized stimulation through the antigen receptor in addition to mercury exposure to examine signal transduction pathway alterations. The question our study attempts to answer is what effect longer exposure times with lesser amounts of mercury have on signal transduction pathway molecules in a B and T lymphocyte cell line. It is also important to note that our work examines the activation status of these molecules and cells in the absence of stimulation through the antigen receptor. We examined the effects of mercury exposure alone on the cells.

To expand upon the studies performed by McCabe et al., we investigated the activation of EL4 T lymphocytes (H-2^b) and WEHI-231 B lymphocytes (H-2^d) signal transduction molecules using mercury concentrations of 0.01, 0.1 and 1 μ M HgCl $_2$. Both lines are of an MHC haplotype susceptible to mercury stimulation of autoimmunity. The results of mercury on signaling molecules can be measured by the use of the phosphoflow method, wherein flow cytometric analysis of levels of phosphorylation of internal

signaling molecules is examined and quantified (Gill et al. 2014, Goldeck et al. 2013, Toapanta et al. 2012). To expand upon the exposure times used in the previously published studies, cells were incubated in mercuric chloride-laced media for either 12 (acute) or 96 (chronic) hours. Under these conditions, our findings indicate mercury alone causes significantly increased phosphorylation of several signal transduction molecules.

Materials and Methods

Cells

EL4 T cells or WEHI-231 B cells (obtained as a generous gift from Randy Hardy, Fox Chase Cancer Center) were cultured in RPMI-1640 media supplemented with 5% FBS, L-glutamine, Penicillin and Streptomycin, HEPES buffer and 2-mercaptoethanol. Cells were maintained in a 5% CO $_2$ incubator at 37°C. Trypan blue exclusion dye was used to ensure viability (~90%).

HgCl $_2$ Necrosis Experiments

EL4 and WEHI-231 cells were incubated in multiple concentrations (0 μ M, 0.01 μ M, 0.1 μ M, 1 μ M and 5 μ M) of HgCl $_2$ in the media for either 12 or 96 hours. Cells were harvested, pelleted at 1000 x g for 8 minutes and washed once in 1X Phosphate-Buffered Saline (PBS). Cells were resuspended in 10 μ g/mL propidium iodide and analyzed via flow cytometry to determine toxicity.

Antibodies

AlexaFluora488-labeled Zap-70(pY319)/Syk (pY352), PE-labeled ERK1/2(pT202/pY204), PE-labeled p38MapK (pT180/pY182) and PE-labeled PKC- α (pT638) were purchased from BD Bioscience laboratories.

Phosphospecific Flow Cytometry

Cells were incubated in HgCl $_2$ -laced media for either 12 or 96 hours. EL4 cells were exposed to 1 μ M HgCl $_2$ whereas WEHI-231 cells were exposed to 0.01 μ M, 0.1 μ M and 1 μ M HgCl $_2$. Density gradient centrifugation with Lympholyte-M ensured that only living cells were harvested and subjected to intracellular staining. Cells were fixed for 15 minutes with 1% paraformaldehyde, pelleted at 1000 x g and washed with PBS. Cell membranes were permeabilized using 1 ml ice-cold 100% v/v methanol for 10 minutes. Following permeabilization, 1 ml PBS was added to rehydrate the cells. Cells were pelleted at

1000 x g and washed an additional two times with PBS. Subsequently cells were exposed to 20 μ l antibody suspended in 30 μ l staining buffer (5% FBS in PBS) for 30 minutes on ice in total darkness, as per the manufacturer's protocol. Cells were washed once in PBS and resuspended in 1 ml staining buffer for analysis. The level of fluorescent antibody within the cells was detected using 488nm laser on an analytical flow cytometer (Partec GMmbH, Munster, Germany). FCS Express software (DeNovo Software, Los Angeles, CA, USA) was used to determine intensity of the fluorescence in each sample.

Statistics

All statistics were performed using JMP Statistical Analysis Software (S.A.S., Cary, NC, USA). For all EL4 data, means were compared using Student's t-tests assuming unequal variances. For all WEHI-231 data, means were compared using two-way ANOVA for block design. Tukey HSD *post hoc* analysis was performed following a significant finding. All data were expressed as means $\pm$ 1 standard error and significance was taken as $p < 0.05$.

Results

Toxicity of HgCl₂ in EL4 and WEHI-231

Individual cell lines were incubated in HgCl₂-laced media at specified concentrations for either 12 or 96 hours. Cells were removed from HgCl₂-laced media and stained with propidium iodide to detect dead cells. Cell death studies indicated an increase in death numbers following exposure to HgCl₂, but it was shown that viable cells were still present within each sample (Table 1).

Table 1. Percent living cells following incubation in mercury-laced media for either 12 hours (acute) or 96 hours (chronic). Data are average of three replications with standard error indicated.

HgCl ₂ (μ M)	WEHI-231		EL4	
	Acute	Chronic	Acute	Chronic
0	98.6(+/-0.6)	97.8(+/-1.7)	93.4(+/-1.9)	89.5(+/-1.9)
0.01	70.7(+/-1.5)	84.2(+/-1.7)	29.7(+/-1.4)	21.2(+/-1.9)
0.1	61.6(+/-2.1)	81.7(+/-2.6)	15.8(+/-0.8)	13.1(+/-0.5)
1	40.5(+/-2.5)	67.3(+/-2.3)	9.38(+/-0.98)	9.3(+/-1.4)
5	32.2(+/-1.1)	40.5(+/-0.95)	4.92(+/-0.49)	5.7(+/-0.8)

Inappropriate Activation in EL4 T cells

EL4 T cells were incubated for either 12 or 96 hours in HgCl₂-free media and 1 μ M HgCl₂-laced

media. Analytical flow cytometry was used to detect antibodies bound to phosphorylated forms of signaling molecules ERK1/2, PKC α , and p38MAPK. In the acute treatments, all molecules had significantly higher mean signal strength when compared with control samples (Figure 1).

The preliminary studies of EL4 T cells included 4 treatment groups (0 μ M, 0.01 μ M, 0.1 μ M and 1 μ M HgCl₂). During these studies we found 0.01 μ M and 0.1 μ M HgCl₂ did not exhibit any deviation from the results of the negative control while 1 μ M HgCl₂ showed dramatic change (results for 0.01 μ M and 0.1 μ M not shown). For this reason, the lower concentrations were not tested further.

Chronic treatment with 1 μ M HgCl₂-laced media resulted in a significant increase in mean signal for pp38MAPK, but signal from 1 μ M HgCl₂-treated cellular pPKC α and pERK1/2 were not found to be significantly higher than control treatments (Figure 2). This finding is likely due to a high degree of variation between replicates.

Inappropriate Activation in WEHI-231 B cells

WEHI-231 B cells were incubated for either 12 or 96 hours in HgCl₂-free, 0.01 μ M, 0.1 μ M and 1 μ M HgCl₂-laced media. Analytical flow cytometry was used to detect antibodies bound to phosphorylated forms of signaling molecules SYK, ERK1/2, PKC α , and p38MAPK. SYK is an important tyrosine kinase in the B cell receptor signaling pathway upstream of ERK (Gill et al. 2014, Rosenspire and Stemmer 2010).

In the acute treatments, pSYK, pPKC α , and pp38MAPK were found to have significant differences in mean signal strength between treatments. For these three molecules, the negative control and 0.01 μ M HgCl₂ signals were not different from one another; however signal strength from 1 μ M HgCl₂ treatments were significantly elevated relative to the lower concentrations (Figure 3).

Although there was no significant difference in the treatments for pERK1/2, the trend of elevated signal strength in higher concentrations is present (Figure 3; Tukey HSD, levels not connected by same letter are significantly different).

In chronic treatments, all four signaling molecules exhibited significant differences in signaling strength within each group. In pSYK and pERK1/2, 0.1 μ M and 1 μ M HgCl₂ showed an increase in signal strength over the negative control and 0.01 μ M HgCl₂. The same elevated trend can be seen for pPKC α and pp38MAPK, however *post hoc* analysis did not find significant differences between treatments - this finding is likely

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due to a high degree of variation between replicates (Figure 4; Tukey HSD, levels not connected by the same letter are significantly different).

Figure 1. Fluorescent signal strength of three EL4 signal transduction molecules following 12 hour mercury exposure. Data shown are means $\pm$ 1 SE; n = minimum 5. Statistically significant differences observed in p-ERK1/2, p-PKCα, and p-p38MAPK levels upon treatment with mercuric chloride, indicating an increase in signaling through mercury exposure alone.

Figure 2. Fluorescent signal strength of three EL4 signal transduction molecules following 96 hour mercury exposure. Data shown are means $\pm$ 1 SE; n=5. Statistically significant difference in p-p38MAPK levels only upon mercury exposure, indicating less of a long-term effect of mercury upon signaling molecule status.

Discussion

Antigen binding draws receptor molecules into close proximity and induces cross-linking that activates the cell. This assemblage of signaling molecules is described as the B cell signalosome (in B cells). It is not fully understood, but is generally accepted to occur upon activation through antigen receptors (Gill et al. 2014, Rosenspire and Stemmer 2010). Activated

Figure 3. Fluorescent signal strength of four WEHI-231 signal transduction molecules following 12 hour mercury exposure. Data shown are means $\pm$ 1 SE; n=3. Means not sharing the same notation are significantly different. No statistical difference was observed between treatment in pERK1/2 levels upon mercury exposure.

Figure 4. Fluorescent signal strength of four WEHI-231 signal transduction molecules following 96 hour mercury exposure. Data shown are means $\pm$ 1 SE; n=4. Means not sharing the same notation are significantly different. Analysis showed an overall significant difference in phosphorylation for p-PKCα or p-p38MAPK, but no statistical difference between treatments was observed for p-PKCα or p-p38MAPK.

lymphocytes then differentiate into effector cells that act to clear out the antigen using a variety of mechanisms. This experiment shows that mercury is causing activation signal transduction events in the absence of antigen or artificial stimulation simulating an antigen. At this time, the mechanism by which this increased activation is occurring is unknown. It is suspected that mercury, which has an affinity for sulfhydryl groups, is binding to the sulfhydryl-rich receptors on the surface of lymphocytes (Mason et al. 1995). This may bring the receptors and their attached Src family kinases into close proximity, allowing

autophosphorylation to begin the signal transduction cascade (Figure 5).

Figure 5. (A) Illustration of a lymphocyte in the absence of mercury. Surface receptors are separated and kinases are not in close enough proximity to autophosphorylate. (B) Illustration of a lymphocyte in the presence of mercury binding to sulfhydryl groups on cysteine amino acids in receptors. Binding may draw the receptors near and allow autophosphorylation events to occur between Src family kinases, including SYK.

It has been known for decades that mercury binds to sulfur atoms found in sulfhydryl groups on the amino acid cysteine (Boyer 1954). Until recently, the majority of mercury binding research focused on chemical interactions rather than the biological implications. Among several more recent works, one paper looked at how mercury binds to and alters the structure of human serum albumin (Li et al. 2007, Haase et al. 2015, Bal et al. 2013).

This brings about an interesting question related to the experiments performed here and elsewhere: If serum albumin can bind mercury, does the presence of the protein in tissue culture medium change the interpretation of the results presented here and in other studies? Tissue culture methods also frequently use 2-mercaptoethanol for support of cells, which exhibits capacity for binding mercury as well.

Answering this question is somewhat difficult. A study on zinc adsorption to serum proteins indicates that the ions can be largely soaked-up by the proteins in vitro. This phenomenon is characterized as a metal ion buffering capacity (Haase et al. 2015). Jurkat T cells, Raw 264.7, BV-2, and L929 cells were each examined for toxicity induction by zinc ion concentrations in increasing concentrations of fetal calf

serum and, separately, with differing amounts of bovine serum albumin. These studies indicated a decrease in cytotoxicity for each cell line exposed to zinc ions as culture protein levels were increased. Results also indicated a decrease in free ion concentration as protein levels increased in the medium. The authors cite several studies indicating that zinc ions are capable of inducing toxicity in cultured cells at nanomolar concentrations (Colvin et al. 2010, Schmidt et al. 2010, Bozym et al. 2010).

While this is an important finding, it may not be directly relevant to the results of our study. Mercury and zinc ions, while chemically related, are still different. They may be bound differentially by serum proteins, and the Haase study didn't extensively examine effects of mercury specifically. While several citations in the Haase study relate to other possible binding/depletion possibilities for zinc ions, these references don't address such concerns for mercury. A software package is available and was used in the Haase study for examination of zinc speciation, but mercury speciation and levels in different circumstances were not addressed to the same level of detail. It is unclear if lipids or phosphates would have an effect on media mercury levels as they do for zinc (Lu et al. 2012, Verweij 2012). It is also worth noting that only the Jurkat line is lymphocytic in nature. While it is lymphocytic, it is also a human (not murine) T cell line. Our studies, and those of McCabe, Gill, and Rosenspire, utilized murine lines, and reported more extensively on the B cell line results than the T cell line. The other three murine cell lines used in the zinc studies are macrophage, microglial, and fibroblast in cellular origin, respectively. Effects on one cell lineage may not be directly applicable to others.

Another study examined effects of mercury on WEHI 231 cells in the presence or absence of 2-mercaptoethanol (McCabe et al. 1999). The results of this study may also not be directly relevant to our particular methods for two reasons. The first is that this study utilized stimulation in vitro through the antigen-receptors on WEHI 231 cells via anti-Igμ antibodies. We did not use stimulation in our studies, relying instead on the effects of mercury alone. Second, while the concern about the presence of 2-mercaptoethanol and fetal bovine serum possibly acting as a metal ion buffer may be accurate, examination of several studies indicate neither is removed from the tissue culture methods used (Gill et al. 2014; McCabe et al. 1999; McCabe et al. 2007).

While we cannot answer definitively whether the presence of 2-mercaptoethanol and fetal bovine serum

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may act as a metal ion buffer in our studies, it may not matter. Previously published work has also used both compounds in similar experiments. Even more interesting is the idea at hand – if either compound sequesters mercury ions, effectively reducing their levels available for effects on the cell lines being cultured, it could mean that even lower doses of mercury actually reaching cells can alter signal transduction events in murine lymphocytes *in vitro*. The impact of that speculative idea on *in vivo* situations could be very intriguing indeed.

The most interesting finding in our study was that the acute treatment of the WEHI-231 B cells exhibited a dose-dependent response, in which increasing HgCl_2 concentrations resulted in more activation. While this result is probably to be expected, it has not been illustrated in work to this point. This dose-dependent response was not seen under chronic exposure – instead, there seems to be a threshold between $0.01\ \mu\text{M}$ and $0.1\ \mu\text{M}$ HgCl_2 in which signaling dramatically increases. This may be due to the fact that $0.01\ \mu\text{M}$ HgCl_2 is not a high enough concentration to affect WEHI-231 B cells, whereas $0.1\ \mu\text{M}$ HgCl_2 sufficiently draws B cell receptor complexes together to initiate the activation cascade. It could also be that the signaling molecules involved have become saturated and less responsive to continued exposure to mercury, requiring an even higher dose to initiate new signaling events. It is important to note that previous studies have not examined effects on signaling molecules following this length of *in vitro* mercury exposure. This represents a novel finding.

Most previous studies use a method for stimulating lymphocytes that simulated antigen/APC binding to artificially begin the signaling cascade (McCabe et al. 1999, Ziembra et al. 2009). In concurrence with these studies, we initially used anti-CD3 and anti-IgM antibodies to artificially stimulate EL4 and WEHI-231 cells, respectively. However, with increased HgCl_2 exposure time, it was not possible to differentiate between background stimulation due to HgCl_2 and stimulation due to the antibodies (results not shown).

Possible future research directions include moving into *in vivo* experiments using mice with an H-2^s background that would mimic a human immune system with a genetic predisposition to developing autoimmunity. Mice will metabolize HgCl_2 in a manner similar to humans which may result in different effects on immune cells than what is seen in this *in vitro* study. Closer examination of mRNA and/or protein levels for signaling molecules in acute

and chronic mercury exposure may also shed greater light on the effects observed.

Since it is not possible to prevent human exposure to environmental mercury compounds, it is necessary to gain insight into treatment and prevention of mercury-induced diseases. This study, and future work, will help us to characterize the effects of mercury at the molecular level and possibly find the mechanism(s) by which mercury causes diseases of the immune system.

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