Cultured lymphocytes from autistic children and non-autistic siblings up-regulate heat shock protein RNA in response to thimerosal challenge

Stephen J. Walker a,*, Jeffrey Segal, Michael Aschner b

a Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC 27156, United States
b Departments of Pediatrics, Pharmacology, and the Kennedy Center, Vanderbilt University Medical Center, 1162 21st Avenue South, B-3307, Medical Center North, Nashville, TN 37232-2495, United States

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Abstract

There are reports suggesting that some autistic children are unable to mount an adequate response following exposure to environmental toxins. This potential deficit, coupled with the similarity in clinical presentations of autism and some heavy metal toxicities, has led to the suggestion that heavy metal poisoning might play a role in the etiology of autism in uniquely susceptible individuals. Thimerosal, an anti-microbial preservative previously added routinely to childhood multi-dose vaccines, is composed of 49.6% ethyl mercury. Based on the levels of this toxin that children receive through routine immunization schedules in the first years of life, it has been postulated that thimerosal may be a potential triggering mechanism contributing to autism in susceptible individuals. One potential risk factor in these individuals may be an inability to adequately up-regulate metallothionein (MT) biosynthesis in response to presentation of a heavy metal challenge. To investigate this hypothesis, cultured lymphocytes (obtained from the Autism Genetic Resource Exchange, AGRE) from autistic children and non-autistic siblings were challenged with either 10 μM ethyl mercury, 150 μM zinc, or fresh media (control). Following the challenge, total RNA was extracted and used to query “whole genome” DNA microarrays. Cultured lymphocytes challenged with zinc responded with an impressive up-regulation of MT transcripts (at least nine different MTs were over-expressed) while cells challenged with thimerosal responded by up-regulating numerous heat shock protein transcripts, but not MTs. Although there were no apparent differences between autistic and non-autistic sibling responses in this very small sampling group, the differences in expression profiles between those cells treated with zinc versus thimerosal were dramatic. Determining cellular response, at the level of gene expression, has important implications for the understanding and treatment of conditions that result from exposure to neurotoxic compounds.

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1. Introduction

Autism is defined as a specific clinical syndrome subject to the following criteria: (a) impairments in social relatedness; (b) impairment of speech and/or language; (c) restricted, repetitive, and stereotyped patterns of behaviors, interests, and activities. The number of families seeking referrals for social services for autistic children has risen significantly in recent years. In 2004, the CDC issued an “Autism Alert”, announcing that the reported prevalence of autism spectrum disorders had risen to alarming levels, currently affecting approximately one in every 166 American children (Anon., 2006). There is an ongoing debate about whether these increases are related to improved diagnosis or actual increases in prevalence. At the same time, many parents have reported that regression into autism occurs shortly after injection with any number of childhood vaccines—including hepatitis B, DPT, or MMR. Adding to the controversy is the observation that, until recently, many childhood vaccines manufactured as multi-dose vials were preserved with thimerosal, a compound that is ~50% ethyl...
mercury. Even now, some influenza vaccines still contain thimerosal. Based on the observation that many of the clinical symptoms of mercury toxicity are similar to the clinical picture of autism, a hypothesis was advanced that suggested autism rates are rising as a result of a vaccine schedule that injects infants with an excessive amount of organic mercury at a developmentally sensitive stage in life (Ball et al., 2001; Bernard et al., 2001, 2002).

Although there is ample evidence that autism is heritable (Bailey et al., 1995; Le Couteur et al., 1996; Fombonne et al., 1997; Piven et al., 1994, 1997), it is improbable that the increased prevalence is the result of an isolated genetic etiology. A genetic predisposition, however, combined with a novel environmental insult that unmask an individual’s susceptibility, could help explain phenotypic expression and rising numbers. If an environmental toxin such as thimerosal is associated with autism, it is difficult to reconcile that most children appear resistant to equivalent doses of the insult. A plausible explanation is that there are individuals who are uniquely susceptible, either through genomic and/or genetic influences, through multiple insults, or through some combination of factors, namely gene–environment interaction. One fertile area of investigation is the regulation and expression of a group of proteins responsible for the body’s biological response to heavy metals, the metallothioneins (MTs). Metallothioneins are a class of cysteine-rich proteins that function to transport heavy metals for detoxification purposes. The expression of MTs is inducible by numerous environmental and stress-related factors (e.g. Park et al., 2001; Tandon et al., 2001). Indeed, their presence in astrocytes prevents accumulation of damaging metals in adjacent neurons (Aschner et al., 1996, 1997). When the quality or quantity of MT is impaired, clinical symptoms can be manifested at lower levels of toxic metal exposure. For example, genetic knockout mice for an MT gene are more susceptible to heavy metal toxicity (Liu et al., 1999, 2000a,b). Conversely, mice with extra copies of MT have extra protection against mercury toxicity (Yao et al., 1999, 2000).

Human white blood cells express MT genes of most functional isoforms at baseline and in response to cadmium or zinc induction (Harley et al., 1989; Ganguly et al., 1996; Vandeghinste and Proost, 2000). Human lymphocytes also produce metallothionein when treated with mercury (Yamada and Koizumi, 1991). This study examines the quantitative dynamics of MT production in lymphocytes in response to thimerosal in autistic patients versus controls. The control lymphocytes are derived from unaffected siblings, those without phenotypic expression of the clinical picture of autism.

Our working hypothesis is that children with regressive autism are unable to produce qualitatively or quantitatively “normal” MT proteins because of either some genetic anomaly or due to an inability to up-regulate production of MT in response to heavy metal exposure. Inadequate protection in this subpopulation from toxins such as mercury (thimerosal) leads to a heavy metal burden that contributes to clinical regressive autism.

2. Materials and methods

2.1. Materials

Plasticware, including Falcon® 15 and 50 ml conical tubes and Falcon® T-25 and T-75 cell culture flasks were purchased from Becton Dickinson (through Fisher Scientific, Pittsburgh, PA). Chemicals including formamidine, formaldehyde, thimerosal (ethylmercurithiosalicylic acid sodium salt) and zinc chloride were obtained from Sigma–Aldrich Chemical Corp. (St. Louis, MO). RPMI-1640 media was purchased from Gibco (Rockville, MD) and RNA Stat-60 came from Tel-Test (Friendwood, TX). FBS, l-glutamine, and penicillin/streptomycin were obtained from the Wake Forest University School of Medicine Cell Culture Facility.

2.2. Cell culture

Two populations of Epstein Barr Virus (EBV)-immortalized B lymphocytes, from autistic and non-affected siblings, representing three pedigrees (Fig. 1) were used for these studies. Eleven vials of cells, obtained from the Autism Genetic Resource Exchange (AGRE, Los Angeles, CA; Geschwind et al., 2001) as cryo-preserved stocks were warmed in a 37 °C water bath until

![Figure 1](image_url)

Fig. 1. Pedigrees of the families. Three multiplex families with at least one autistic child and unaffected sibling(s) were selected for this study. Only lymphocyte cultures derived from the 11 children were used. Family A has two diagnosed autistic children (1 M and 1 F), and one unaffected child (M); family B has one diagnosed autistic child (F), one child (F) diagnosed with pervasive developmental disorder (PDD), and two unaffected children (1 M and 1 F); family C has one older, unaffected child (M), and triplets (3 M), two of whom are diagnosed autistic. Symbols: circles (○) represent females; squares (□) represent males. Open symbols represent unaffected members, closed symbols represent autism diagnosis, and shaded symbol represents PDD diagnosis. Regular numerals indicate family order—1: mother; 2: father; 3–6: children; italic numerals indicate specific disease traits—1: verbal; 2: non-verbal; 3: regressive.
ice crystals just disappeared and then transferred to 15 ml conical tubes containing 10 ml of pre-warmed RPMI-1640 media (to dilute the DMSO). Lymphocytes were triturated and then spun in a clinical centrifuge at 275 × g for 5 min. The resultant supernatant was removed and discarded, and the cells were resuspended in 10 ml of fresh growth media (RPMI-1640 supplemented with 15% FBS, 1-glutamine, and penicillin/streptomycin). Cells and media were transferred to T-25 flasks, where they were maintained at 37 °C and 5% CO2. Cells were fed by replacing approximately half of the media every 2–3 days and subcultured as confluency dictated, typically at 3–4 day intervals (when cells were at >70% confluency).

2.3. Challenge experiments

The thimerosal treatment solution was prepared as a 2× stock (20 μM thimerosal) in RPMI-1640 media (plus supplements). The zinc treatment was also prepared as a 2× stock (300 μM zinc chloride) in media plus supplements. For the challenge experiments, individual cell cultures (3× flasks) were grown to near-confluency and (approximately) equal numbers of cells, in a volume of 8 ml, were added to individual T25 flasks. Next, 8 ml of media containing either thimerosal (to a final concentration = 10 μM), zinc (to a final concentration = 150 μM), or media only (=control) was added and the flasks were incubated for 6 h at 37 °C under 5% CO2. Following incubation the flask contents were transferred to 50 ml conical tubes and centrifuged at 275 × g for 5 min. Cell culture pellets were decanted (by pouring the media off) and stored at −20 °C.

2.4. RNA Isolation and QC

For each experimental sample total RNA was isolated from the equivalent of 8 ml of cells (as frozen pellets from the challenge experiments) using RNA Stat-60 according to the manufacturer’s instructions. Briefly, 1 ml of reagent was added directly to the cell pellet and following cell lysis and processing the resulting RNA pellet was resuspended in 11 μl nuclease-free water.

RNA concentration and quality were estimated spectrophotometrically and by electrophoresis on denaturing formaldehyde-agarose gels. A260/A280 ratios of the RNA were measured using 100-fold dilutions of RNA in pH 8.0 TE buffer, with a Beckman DU-640 spectrophotometer (Beckman Coulter). Inclusion criteria were two A260/A280 readings between 1.8 and 2.0, with no more than 5% deviation between readings. To determine if the RNA was largely intact, ~1 μg of RNA was brought to 7 μl with nuclease-free water, mixed with 7 μl of denaturing solution (200 μl formamide, 80 μl formaldehyde, 30 μl 10× MOPS, and 15 μl 1% ethidium bromide), and incubated at 65 °C for 5 min. The samples were run in 1× formaldehyde-agarose running buffer on a 1% agarose-formaldehyde gel at 50 V for 30 min using a RunOne™ Electrophoresis System (EmbiTec). Bands were visualized on a UVP ImageProcessor 7500 gel doc (Ultra Violet Products), and resultant images were processed with TINA (Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany).

RNAs (~10 μg each) prepared in this fashion and derived from two individuals, one autistic and a non-autistic sibling (six RNAs total: (1) autistic – thimerosal treated; (2) autistic – zinc treated; (3) autistic – control; (4–6) the corresponding three non-autistic samples) were then delivered to a core facility for microarray analysis.

2.5. Expression profiling using affymetrix arrays

Total RNA from the two individuals, one autistic and a non-autistic sibling, tested under three treatment conditions (thimerosal, zinc, and control) – six samples total – were supplied to a commercial Affymetrix microarray facility (Genome Explorations Inc., Memphis, TN) for processing. For these experiments the Human Genome U133A Gene-Chips® were used. These microarrays contain ~500,000 unique oligonucleotide features representing 18,400 transcripts and variants. Labeling, hybridization, washing, and signal generation and detection were all performed according to the manufacturer’s protocols using reagents supplied by the facility. A total of six chips were run, one for each sample. Upon completion of the array experiment at Genome Explorations Inc. datafiles (five per microarray: CHP, DAT CEL, TXT, RPT) were supplied to the investigators for analysis. The final signal output and “present” calls were generated using Affymetrix MAS software v5.0.

2.5.1. Raw data signal processing and normalization

The image files that are created from array hybridization and scanning were loaded into the Affymetrix MAS 5.0 program (Microarray Analysis Suite 5), and after visual inspection of quality, were processed to extract signal intensities. The arrays were normalized using the global scaling method according to standard Affymetrix analysis procedures. This scaling corrects for differing overall intensities across the arrays resulting from differences in lab experimental assay variables, which are independent of relative transcript concentration in the tissues. All arrays were scaled to the same global target intensity values, and the intensity scaling factors checked to ensure that they only vary within a restricted (small) range. Scaling all of the arrays to the same target intensity permits the comparison of any array, or group of arrays, to any other.

2.5.2. Detection of transcript presence

After each array was normalized (scaled), MAS 5.0 was used to detect the presence or absence of each array probe set transcript within the RNA used for the hybridization. The standard Affymetrix signal detection algorithm was applied to the probe set signals, to assign an absolute detection call of Present, Marginal, or Absent for each probe set for the array. The detection calls are based on statistical p-values associated with each probe set signal.

2.6. Microarray data analysis

Microarray data were analyzed using GeneSifter™ (http://www.genesifter.net/), a web-based software suite for microarray
analysis. For these analyses, the software was used with normalization set to “off” so that fluorescence values could be compared directly. The groups (a: autistic + zinc versus autistic + media only; b: autistic + thimerosal versus autistic + media only; c: non-autistic + zinc versus non-autistic + media only; d: non-autistic + thimerosal versus non-autistic + media only) were then compared, using a fold-change cutoff ratio of 1.5, to generate a list of genes that were up or down-regulated in each comparison. No statistical analyses were possible with only a single microarray in each group.

A second set of analyses was performed by combining the control datasets, the thimerosal datasets and the zinc datasets and then comparing: (1) zinc treatment (combined) versus control (combined) and, (2) thimerosal treatment (combined) versus control (combined). The combined data were analyzed by t-test, using a fold-change of 1.5 and p ≤ 0.05, to generate a list of genes that were significantly up or down-regulated.

3. Results

3.1. Time course and dose response experiments

In an effort to determine an appropriate dose and time course for the thimerosal challenge experiments several preliminary assays were performed. First, replicate flasks of cells were dosed with either: 0, 5, 10, 25 or 50 μM thimerosal for 6 h. Following dosing, total RNA was isolated from the cells and examined by gel electrophoresis for RNA integrity. For 0, 5 and 10 μM thimerosal-treated samples, the RNA appeared to be largely intact with very little evidence of degradation. At 25 μM thimerosal, however, the RNA appeared to be somewhat degraded (no longer evidence of distinct 28S band; data not shown) and at 50 μM the RNA appeared to be further degraded.

The second set of preliminary assays was performed to determine an appropriate incubation time. For these experiments, replicate flasks of cells were dosed with 10 μM thimerosal and incubated for either 6 or 24 h. Following incubation, RNA was isolated and evaluated and found to be completely degraded at 24 h (data not shown). Based on results from these pilot experiments, the assay conditions chosen to effect maximum response without cell death were a challenge with 10 μM thimerosal for 6 h.

3.2. Zinc treatment and gene expression analysis on individual arrays

When lymphocytes from autistic and non-autistic individuals were treated with zinc, and total RNA was then examined by microarray, there were between ~8400 and ~9400 genes that were different by a ratio ≥1.5-fold (Table 1). In the autistic sample, almost twice as many genes were down-regulated (6131 versus 3260 up-regulated), whereas in the non-autistic zinc-treated lymphocytes there were many more up-regulated transcripts (5017 compared to 3367 down-regulated).

The gene lists were sorted by fold-change and in both sample comparisons the most abundant family of genes that was up-regulated was the metallothionein gene family, with at least nine different transcripts (MT 1K, MT 1F, hMT-1F, MT 1X, MT 1L, MT 1H, MT-1E, MT 2A, and MT 1G) being turned on or up-regulated in the two lymphocyte populations.

3.3. Thimerosal treatment and gene expression analysis on individual arrays

Lymphocytes from autistic and non-autistic individuals treated with thimerosal showed a greater overall number of differentially expressed genes than the zinc-treated cells (~11,220 and ~10,200, respectively; Table 1). In these comparisons, a much larger percentage of genes were down-regulated in both samples.

When gene lists from thimerosal-treated cells were sorted by fold-change, there were no MT genes differentially regulated however there were a number of heat shock proteins up-regulated in both comparisons (heat shock 70 kDa protein 6, HSP70B, heat shock 70 kDa protein 1A, heat shock protein 47, and heat shock 70 kDa protein 1B).

3.4. Gene expression analysis using combined datasets

The comparison of microarray results from individual samples yielded some compelling trends between gene expression responses following treatment with either zinc or thimerosal: however the between-individual (autistic and non-autistic siblings) gene expression changes did not appear to yield striking differences. Therefore, a final analysis was performed by combining the array data from the individuals and then comparing treatment groups. Treatment of lymphocytes with zinc resulted in 795 genes that were differentially changed (fold-change ≥1.5; p ≤ 0.05), with an approximately equal number being up- and down-regulated (Table 1). When the analysis criteria were made more stringent (fold-change ≥1.5; p ≤ 0.01) there were 322 differentially expressed genes in this comparison. Sorting this list by p value revealed 9 of the first 11 genes in the list are MTs and all 9 are significantly up-regulated (Fig. 2; Supplemental Table 1). Thimerosal treatment of lymphocytes resulted in a much larger number of genes (1800) that were significantly changed, with two thirds of those representing down-regulated transcripts. The composite gene lists, sorted by fold-change, look very similar to the individual comparisons, and they reveal, even more clearly, the differential

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
<th>Total</th>
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<tr>
<td>Autistic lymphocytes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Thimerosal vs. control</td>
<td>4213</td>
<td>7008</td>
<td>11221</td>
</tr>
<tr>
<td>Zinc vs. control</td>
<td>3260</td>
<td>6131</td>
<td>9391</td>
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<tr>
<td>Non-autistic lymphocytes</td>
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<tr>
<td>Thimerosal vs. control</td>
<td>4232</td>
<td>5926</td>
<td>10158</td>
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<tr>
<td>Zinc vs. control</td>
<td>5017</td>
<td>3367</td>
<td>8384</td>
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<td>Combined</td>
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<tr>
<td>Thimerosal vs. control</td>
<td>546</td>
<td>1254</td>
<td>1800</td>
</tr>
<tr>
<td>Zinc vs. control</td>
<td>382</td>
<td>413</td>
<td>795</td>
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</tbody>
</table>
The primary goal of this study was to investigate the hypothesis that some autistic individuals may be uniquely susceptible to heavy metal toxicity. This could be the result of an inability to mount an adequate cellular response, for example an inability to up-regulate the production of metallothioneins, when challenged with heavy metals in the environment. To test this hypothesis lymphocytes in culture, derived from autistic and non-autistic (sibling) children, were exposed to either zinc (zinc chloride) or thimerosal (ethylmercurithiosalicylic acid) response to the two different metal challenges (Fig. 3; Supplemental Table 2).

4. Discussion

The primary goal of this study was to investigate the hypothesis that some autistic individuals may be uniquely susceptible to heavy metal toxicity. This could be the result of an inability to mount an adequate cellular response, for example an inability to up-regulate the production of metallothioneins, when challenged with heavy metals in the environment. To test this hypothesis lymphocytes in culture, derived from autistic and non-autistic (sibling) children, were exposed to either zinc (zinc chloride) or thimerosal (ethylmercurithiosalicylic acid...
sodium salt). High density microarrays were used to measure gene expression associated with each of these exposures. Although in this small sample size there were no differential responses between the autistic and non-autistic sibling, a larger number of individual samples (biological replicates) must be examined to determine with certainty the relevance of this observation. What was apparent from these experiments, when data from the siblings were combined and then analyzed based on treatment, was that the functional genomic responses to the two metals were quite distinct.

In this study we found, not surprisingly, that cells exposed to zinc responded by up-regulating a large number of MT genes (Fig. 2). The lymphocytes in the present study responded to zinc exposure by up-regulating several hundred genes, with MT
Metallothioneins are a group of small nearly ubiquitous proteins comprised of approximately 30% cysteine residues. MTs have a role in protection against the toxic effects of heavy metals, xenobiotics and γ-irradiation, as well as in drug resistance, homeostasis of essential metals, and free radical scavenging (Miura and Naganuma, 2000). These proteins have been shown previously to be inducible in liver and kidney of animals following exposure to Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Cu$^{2+}$, and Ag$^{2+}$ (Karin and Herschman, 1981). Cells in culture also respond to zinc, for example in HELA cells exposed for 24 h to 0.1 mM Zn$^{2+}$ there was a 35–70-fold induction of metallothionein protein (Karin and Herschman, 1981). Astrocytes treated with zinc also respond by upregulating MT gene and protein expression. Astrocytes, pretreated with 100 μM zinc for 24 h, showed a 2.9-fold increase in MT protein levels and a 5.6-fold increase in MT mRNA levels compared to control astrocytes (Aschner et al., 1998).

Cultured lymphocytes in the present study exposed to thimerosal, under identical conditions to cells exposed to zinc, also responded with the up-regulation of hundreds of genes (Table 1). In this case, MTs were not, as a group, responsive to the challenge (Fig. 3). Many of the several hundred up-regulated genes represent neurodegeneration and apoptotic pathways, while many of the significantly down-regulated genes belong to metabolic and cell signaling pathways. This agrees well with studies by Makani et al. (2002) and Humphrey et al. (2005) that showed evidence for mitochondrial-mediated apoptosis in cells exposed to thimerosal.

Although this study did not measure the breakdown of ethylmercury in cultured lymphocytes, there is evidence in the literature to suggest that ethylmercury may be short-lived in peripheral cells. In a study that examined the breakdown of both ethyl and methylmercury by phagocytic cells, Suda et al. (1992) demonstrated that ethylmercury was readily degraded by human polymorphonuclear leukocytes (PMN), rat PMN, guinea-pig PMN, rabbit PMN, guinea-pig macrophages (M phi), human monocytes and guinea-pig eosinophils, while degradation of methylmercury by these cells was always much weaker. These findings are consistent with studies by Burbacher et al. (2005) demonstrating that thimerosal injected into infant monkeys has a much shorter half-life compared to ingested methylmercury. Under these conditions we would expect ethylmercury to have a more potent effect on MT induction compared to methylmercury, but these studies are beyond the scope of this manuscript.

There is some evidence that pretreatment of cells with zinc and/or antioxidants such as reduced glutathione, may afford a degree of protection against heavy metal toxicity. When astrocytes were exposed to methylmercury (10 μM), following pretreatment with zinc, cells were able to attenuate the cytotoxicity of methylmercury, as evidenced in measures of cell volume, Na$^{+}$ uptake, and K$^{+}$ release, suggesting that astrocytic MT induction offers a means of cellular protection against heavy metal neurotoxicity (Aschner et al., 1998). In another study, astrocytes and neurons cultured from human fetal brain tissues were exposed to various doses of methylmercury for 24 h, and LC$_{50}$ values were determined. Longer exposure times resulted in an increase in the degree of cell damage, however the addition of reduced glutathione (GSH) extracellularly to GSH-depleted cells was able to block methylmercury neurotoxicity. Those studies indicated that human neuronal cells had differential responses to methylmercury and that agents with antioxidant properties provided differential protection, depending on the cell type (Sanfeliu et al., 2001). The authors suggested that the major beneficial effect of GSH could be attributed to its ability to form conjugates with methylmercury, reducing the availability of this metallic compound to cells and facilitating its efflux.

In a subsequent study looking specifically at the neurotoxic effects of ethylmercury on neuroblastoma and glioblastoma cells in culture, both cell lines were found to be susceptible to cytotoxicity, associated directly with a depletion of intracellular GSH. When cells were pretreated with formulations that increased intracellular GSH [glutathione ethyl ester or N-acetylcysteine (NAC)], ethylmercury (15 μM) was no longer cytotoxic (James et al., 2005). The authors suggested that the protective effects of GSH or NAC against mercury toxicity warrants further research of these compounds and mechanisms for potential therapeutic value in individuals exposed to a toxic metal burden.

In summary, the zinc-responsive up-regulation of genes in cultured lymphocytes described in this study, specifically the metallothioneins, has been demonstrated previously in several different cell types including neurons, astrocytes, and neuroblastoma cells in culture. Up-regulation of MT mRNA and protein has been shown to provide protection against cytotoxicity to cells that are subsequently challenged with a toxic metal exposure like methylmercury, indicating that this is one of the body’s defense mechanisms to deal with this type of environmental insult. While most of the previous work has been done on neuronal cell types, we have now shown that this response is also present in peripheral (blood-derived) cells. Lymphocytes in culture exposed to ethylmercury, in contrast to zinc, responded by up-regulating large numbers of genes that are involved in neurodegeneration and apoptotic pathways, while down-regulating genes involved in several metabolic and cell signaling pathways. Moreover, cells exposed to ethylmercury for longer than 6 h showed show a time-dependant decrease in viable RNA, indicating they were experiencing cell death. In a subsequent and expanded study, we will investigate the effect of pretreatment of lymphocytes with zinc or GSH, followed by treatment with ethylmercury, to determine whether the pretreatment affords protection from cytotoxicity in this cell culture model. We will also increase our biological replicate number to allow for a statistically relevant comparison of response to metals between cells derived from autistic children and non-autistic siblings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuro.2006.06.003.

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