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# Thimerosal Neurotoxicity is Associated with Glutathione Depletion: Protection with Glutathione Precursors

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## Abstract

Thimerosal is an antiseptic containing 49.5% ethyl mercury that has been used for years as a preservative in many infant vaccines and in flu vaccines. Environmental methyl mercury has been shown to be highly neurotoxic, especially to the developing brain. Because mercury has a high affinity for thiol (sulfhydryl (-SH)) groups, the thiol-containing antioxidant, glutathione (GSH), provides the major intracellular defense against mercury-induced neurotoxicity. Cultured neuroblastoma cells were found to have lower levels of GSH and increased sensitivity to thimerosal toxicity compared to glioblastoma cells that have higher basal levels of intracellular GSH. Thimerosal-induced cytotoxicity was associated with depletion of intracellular GSH in both cell lines. Pretreatment with 100  $\mu$ M glutathione ethyl ester or N-acetylcysteine (NAC), but not methionine, resulted in a significant increase in intracellular GSH in both cell types. Further, pretreatment of the cells with glutathione ethyl ester or NAC prevented cytotoxicity with exposure to 15  $\mu$ M Thimerosal. Although Thimerosal has been recently removed from most children's vaccines, it is still present in flu vaccines given to pregnant women, the elderly, and to children in developing countries. The potential protective effect of GSH and cysteine against mercury toxicity warrants further research as possible adjunct therapy to individuals still receiving Thimerosal-containing vaccinations.

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**Keywords:** Thimerosal; Neurotoxicity; Glutathione depletion

## INTRODUCTION

Thimerosal (sodium ethylmercurithiosalicylate) was developed by Eli Lilly in the 1930s as an effective bacteriostatic and fungistatic preservative and has been widely used in multidose vials of vaccines and in ophthalmic, otic, nasal, and topical products. Until the removal of Thimerosal from most pediatric vaccines in 2001, the largest human exposure in the US

( $\mu$ g/kg body weight) was in children under 18 months of age undergoing routine childhood immunization schedules. Prior to 2001, a child may have received a cumulative dose of over 200  $\mu$ g/kg in the first 18 months of life (Ball et al., 2001). Although the neurotoxicity of methyl mercury has been relatively well studied, limited information is available on the relative neurodevelopmental toxicity of ethylmercury, the mercury metabolite of Thimerosal. Based on the known toxicity of methylmercury, the cumulative ethylmercury exposure to US pediatric populations in Thimerosal-containing vaccinations was re-examined in 1999 and found to exceed EPA recommended guidelines

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(Ball et al., 2001). Following recommendations by the American Academy of Pediatrics and the US Public Health Service (Public Health Service, 1999), Thimerosal was subsequently removed as a preservative from most children’s vaccines in the US. However, influenza vaccines and Rho D immunoglobulin shots containing Thimerosal are still recommended to pregnant women, and many vaccines given to children in developing countries still contain Thimerosal. The present study was undertaken to better understand the mechanisms underlying Thimerosal toxicity to neurons and astrocytes, the primary CNS targets for organic mercury (Sanfeliu et al., 2001). A better understanding of the neurotoxic mechanism is a necessary prerequisite for the design of intervention strategies for prevention and for the identification of genetic variants that could increase sensitivity to Thimerosal.

Previous mechanistic studies of methylmercury toxicity in astrocytes and neurons have implicated reactive oxygen species (ROS) and depletion of intracellular glutathione as major contributors to mercury-induced cytotoxicity (Sanfeliu et al., 2001). Organic mercury has a high affinity for the thiol (–SH) group on glutathione, a tripeptide composed of cysteine, glutamate, and glycine (Sanfeliu et al., 2003). The cysteine moiety of glutathione carries the active thiol group that binds and detoxifies a variety of heavy metals, including organic and inorganic mercury. Normally, the intracellular concentration of glutathione is extremely high, in the mM range (Meister, 1995); however, with depletion of this essential antioxidant, excess free mercury is available to bind to cysteine thiol groups present in essential cellular proteins, leading to functional inactivation and cytotoxicity.

The synthesis of glutathione in the brain is unique in that brain cells do not express cystathionine gamma lyase, an enzyme in the transsulfuration pathway involved in glutathione synthesis (Awata et al., 1995). As a result, brain cells cannot synthesize cysteine, the rate limiting amino acid for glutathione synthesis. Thus, the brain is dependent on the liver to synthesize and export cysteine for uptake and utilization by astrocytes and neurons for adequate glutathione synthesis (Lu, 1998). In contrast to the brain, the liver expresses the complete transsulfuration pathway from methionine to cysteine and glutathione as diagrammed in Fig. 1. Glutathione synthesized in the liver is exported to the plasma where it is immediately degraded to cysteinylglycine and cysteine (Lu, 1998). Cysteine is converted to cystine in the oxidizing environment of the plasma and subsequently transported to the brain for intracellular glutathione synthesis (Fig. 2).

A second unusual aspect of glutathione synthesis in the brain is the unique metabolic interaction between astrocytes and neurons regarding uptake of cysteine, the rate-limiting amino acid for glutathione synthesis (Dringen and Hirrlinger, 2003; Kranich et al., 1996). Astrocytes and neurons have different affinities for the uptake of oxidized and reduced forms of cysteine for glutathione synthesis (Dringen et al., 2000b). Neurons are unable to take up cystine (oxidized plasma form of cysteine) but can readily transport reduced cysteine for glutathione synthesis. In contrast, oxidized cystine is readily taken up by astrocytes and converted to glutathione as diagrammed in Fig. 2 (Kranich et al., 1998; Wang and Cynader, 2000). Because cysteine is the rate-limiting amino acid for glutathione synthesis, the relative availability

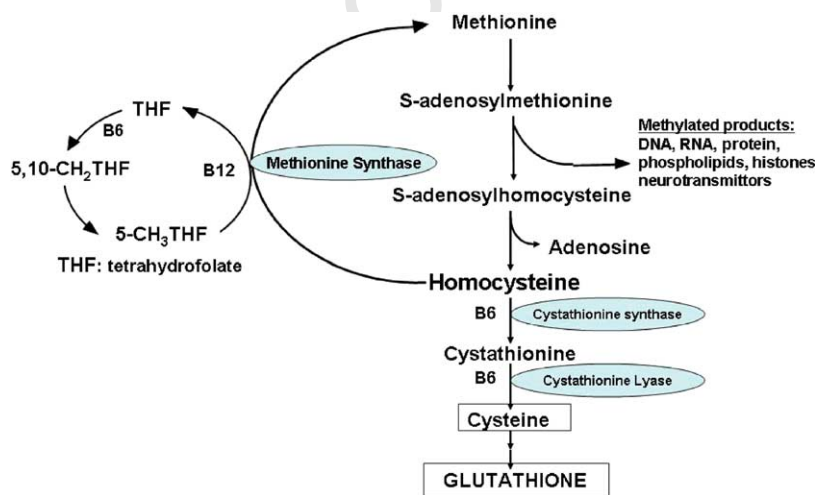


Fig. 1. Pathways of glutathione synthesis. The liver expresses the complete pathway from methionine through homocysteine and cysteine to glutathione. Astrocytes and neurons do not express the enzyme cystathionine lyase and therefore are unable to synthesize cysteine. As a result, astrocytes and neurons are dependent on plasma cysteine derived primarily from the liver to synthesize glutathione.

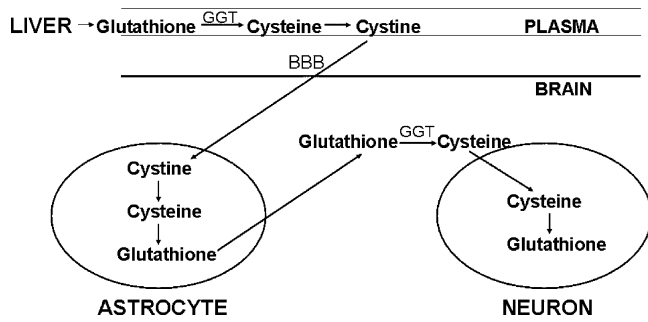


Fig. 2. Interaction between astrocytes and neurons for glutathione synthesis. Glutathione exported from the liver is hydrolyzed to cysteinylglycine and cysteine. In the plasma, cysteine is oxidized to cystine and transported across the BBB and taken up by astrocytes and used to synthesize glutathione. Astrocytes export glutathione into the extracellular space where it is hydrolyzed to cysteine and taken up by neurons for glutathione synthesis. Abbreviations: GGT: gamma glutamyl synthetase; BBB: blood-brain barrier.

of extracellular cystine and cysteine determines intracellular glutathione concentrations and resistance to mercury toxicity in astrocytes and neurons, respectively.

The purpose of the present study was to determine the relative sensitivity of astrocytes and neurons to Thimerosal (ethyl mercury) cytotoxicity in vitro and to determine whether Thimerosal neurotoxicity was associated with depletion of glutathione in cultured human cells as previously reported for methylmercury (Sanfeliu et al., 2001). Acute high dose exposures to Thimerosal ( $\mu\text{mol/L}$ ) in cultured cells were used to study mechanistic aspects of Thimerosal toxicity and not intended to mimic exposures of developing brain cells in vivo to Thimerosal in vaccines (nmol/kg).

## MATERIALS AND METHODS

### Materials

Culture flasks, 96-well plates, and pipettes were obtained from Falcon (Franklin Lakes, NJ, USA). F-12K and MEM culture media were purchased from ATCC (Manassas, VA, USA) and the RPMI 1640 culture media, streptomycin, and Dulbecco's Phosphate Buffered Saline were purchased from Gibco (Grand Island, NY, USA). The TACS™ MTT kit for cell viability assay was purchased from R & D systems (Minneapolis, MN, USA). Fetal bovine serum was purchased from HyClone (Logan, UT, USA). Thimerosal USP, glutathione ethyl ester, cysteine, cystine, *N*-acetyl-L-cysteine, L-methionine, and EDTA-trypsin

were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### Cell Culture

Human neuroblastoma SH-SY5Y CRL 2266 cells and glioblastoma CRL 2020 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in T-25 ml flasks in a humidified incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . The neuroblastoma cells were cultured in 50:50 F-12K media and MEM media containing 15% fetal calf serum and 1% penicillin/streptomycin. The glioblastoma cells were cultured in RPMI 1640 media with supplements recommended for this cell line by ATCC plus 15% fetal calf serum with 1% penicillin/streptomycin.

### Cell Viability Assays

Cell viability before and after Thimerosal exposure was assessed using the MTT assay. Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt, MTT, to insoluble purple formazan crystals at a rate that is proportional to cell viability. For the viability study with Thimerosal exposure, the cultured neuroblastoma and glioblastoma cells were harvested with 0.25% trypsin and replated in 96-well microtitre plates at a concentration of  $6.5 \times 10^5$  cells/ml and  $5 \times 10^5$  cells/ml, respectively, in a  $100 \mu\text{l}$  volume. After a 3-day culture period to reach confluency,  $10 \mu\text{l}$  of Thimerosal in PBS was added to achieve final concentrations of 2.5, 5, 10 or  $20 \mu\text{mol/L}$  in triplicate wells. Pilot studies conducted with increasing duration of exposure at  $37^\circ\text{C}$  indicated that a 48 h incubation period was the threshold for toxicity in the glioblastoma cells whereas only a 3 h incubation was required to achieve similar cytotoxicity in the neuroblastoma cells (data not shown). At the end of the respective incubation periods,  $10 \mu\text{l}$  MTT solution (1:10 v/v) to each well for 1 h followed by  $100 \mu\text{l}$  of dimethylsulfoxide detergent solution to lyse the cells and solubilize the formazan crystals formed in the metabolically active (viable) cells. Triplicate untreated negative control cells were run together with the Thimerosal-treated cells. The optical density (OD) was read in the 96-well plate using the Thermo Max spectrophotometer (Molecular Devices, Sunnyvale, CA) set at 550 nm (with a reference wavelength of 650 nm). Triplicate wells with reagent only served as background controls. The results are expressed as the OD after background subtraction.

## 198 Nutrient Supplementation Studies

199 For the viability studies, cells were exposed to  
 200 15  $\mu\text{mol/L}$  Thimerosal with and without prior incubation  
 201 with *N*-acetylcysteine, cystine, glutathione ethyl  
 202 ester, or methionine at a final concentration of  
 203 100  $\mu\text{mol/L}$  each. *N*-Acetylcysteine is an acetylated  
 204 analog of cysteine that easily crosses the cell mem-  
 205 brane and is rapidly deacetylated inside the cell and  
 206 utilized for GSH synthesis (Zafarullah et al., 2003).  
 207 Glutathione ethyl ester is an esterified form of glu-  
 208 thione that is able to cross the cell membrane against  
 209 the concentration gradient (Anderson et al., 2004).  
 210 Cystine is the disulfide (oxidized) form of cysteine  
 211 that is readily taken up by astrocytes, but not neurons  
 212 (Sagara et al., 1993). Since astrocytes are unable to  
 213 synthesize GSH from methionine, the addition of  
 214 methionine to the media served as a negative control.  
 215 Triplicate aliquots of neuroblastoma and astroglial  
 216 cells were plated in 96-well plates at concentrations  
 217 of  $8 \times 10^5$  cells/ml and  $5 \times 10^5$  cells/ml, respectively.  
 218 The supplements were added to the culture media  
 219 45 min before the addition of Thimerosal and were  
 220 prepared as 10x concentrates and added in 10  $\mu\text{L}$  to the  
 221 cell culture media. Cell viability after Thimerosal  
 222 exposure was assessed with the MTT assay as  
 223 described above. For HPLC analysis of intracellular  
 224 glutathione levels, neuroblastoma and glioblastoma  
 225 cells were plated in triplicate in 6-well plates at a  
 226 density of  $6.5 \times 10^5$  cells/well and  $1 \times 10^6$  cells/well,  
 227 respectively, and cultured for 4 days in order to gener-  
 228 ate sufficient cells for HPLC analysis. Triplicate  
 229 wells were pooled for analysis.

## 230 HPLC Sample Preparation

231 Briefly,  $10^6$  cells were homogenized on ice in  
 232 200  $\mu\text{L}$  of phosphate-buffered saline. To reduce sulf-  
 233 hydryl (thiol) bonds, 50  $\mu\text{l}$  freshly prepared 1.43 M  
 234 sodium borohydride solution containing 1.5  $\mu\text{M}$   
 235 EDTA, 66 mM NaOH and 10  $\mu\text{l}$  iso-amyl alcohol  
 236 was added to the homogenate. After mixing, the solu-  
 237 tion was incubated in a 40  $^\circ\text{C}$  water bath for 30 min  
 238 with gentle shaking. To precipitate proteins, 250  $\mu\text{L}$   
 239 ice cold 10% *meta*-phosphoric acid was added, mixed  
 240 well, and the sample was incubated for 30 min on ice.  
 241 After centrifugation at  $18,000 \times g$  for 15 min at 4  $^\circ\text{C}$ ,  
 242 the supernatant was filtered through a 0.2  $\mu\text{m}$  nylon  
 243 membrane filter (PGC Scientific, Frederic, MD). A  
 244 20  $\mu\text{l}$  aliquot of cell extract was directly injected onto  
 245 the column using Beckman Autosampler (model  
 246 507E).

## HPLC with Coulometric Electrochemical 247 Detection 248

249 The elution of glutathione was accomplished using  
 250 HPLC with a Shimadzu solvent delivery system (ESA  
 251 model 580) and a reverse phase C18 column (5  $\mu\text{m}$ ;  
 252 4.6 mm  $\times$  150 mm, MCM Inc., Tokyo, Japan) obtained  
 253 from ESA Inc. (Chemsford, MA). A 20  $\mu\text{L}$  aliquot of  
 254 cell extract was directly injected onto the column using  
 255 Beckman autosampler (model 507E). The mobile  
 256 phase consisted of 50 mM sodium phosphate mono-  
 257 basic, monohydrate, 1.0 mM ion-pairing reagent  
 258 octane sulfonic acid, 2% (v/v) acetonitrile adjusted  
 259 to pH 2.7 with 85% phosphoric acid, with isocratic  
 260 elution at ambient temperature at a flow rate of 1.0 ml/  
 261 min and a pressure of 120–140 kgf/cm<sup>2</sup> (1800–  
 262 2100 psi). To assure standardization between sample  
 263 runs, calibration standards and reference plasma sam-  
 264 ples were interspersed at intervals during each run. The  
 265 metabolites were quantified using a model 5200A  
 266 Coulochem II electrochemical detector (ESA Inc.)  
 267 equipped with a dual analytical cell (model 5010)  
 268 and a guard cell (model 5020). The concentration of  
 269 intracellular glutathione was calculated from peak  
 270 areas and standard calibration curves using HPLC  
 271 software and expressed per mg protein. An aliquot  
 272 of initial homogenate was used for protein determina-  
 273 tion using the BCA protein assay (Pierce Inc., Rock-  
 274 ford, IL).

## 275 Statistics

276 Values are expressed as the mean  $\pm$  standard devia-  
 277 tion. One way ANOVA was used to determine signifi-  
 278 cant differences between groups and the Bonferroni *t*-  
 279 test for pairwise comparisons using Sigma Stat 2.0  
 280 software. Treatment related differences were consid-  
 281 ered to be significant at  $p < 0.05$ .

## 282 RESULTS

### 283 Dose–Response Characteristics of Thimerosal in 284 Glioblastoma and Neuroblastoma Cells

285 Fig. 3A and B shows the dose response character-  
 286 istics of triplicate cultures of the glioblastoma and  
 287 neuroblastoma cells, respectively. In both cell lines,  
 288 a progressive increase in cytotoxicity (decrease in  
 289 viability) was observed when Thimerosal dose was  
 290 progressively doubled from 2.5  $\mu\text{mol/L}$  to 5, 10 and  
 291 20  $\mu\text{mol/L}$ . Viability was reduced more than 50% in

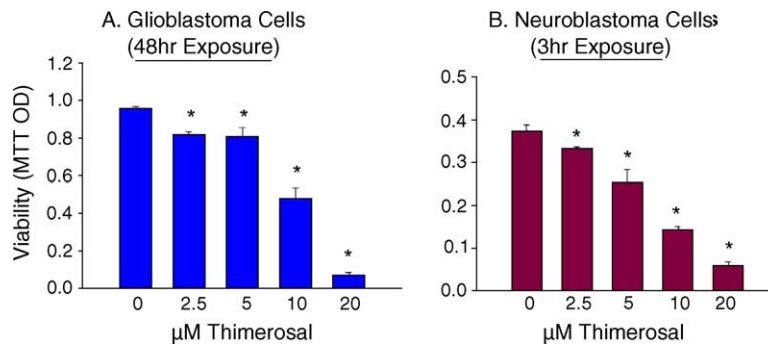


Fig. 3. Viability of glioblastoma cells (A) and neuroblastoma cells (B) with increasing concentrations of Thimerosal in the media. Asterisks indicate significant differences from control cells without Thimerosal treatment ( $n = 3$ ,  $p < 0.01$ ).

292 both cell lines with exposure to 10  $\mu\text{mol/L}$  Thimerosal  
 293 and less than 10% of cells survived a dose of 20  $\mu\text{mol/L}$ .  
 294 Although the shape of the dose response is similar  
 295 between the two cell types, the dose response curve for  
 296 the neuroblastoma cells occurred with a only a 3 h  
 297 exposure whereas the glioblastoma cell required 48 h  
 298 to exhibit similar cytotoxic effects at the same dose.  
 299 These results demonstrate that the neuroblastoma cells  
 300 are much more sensitive to Thimerosal cytotoxicity than  
 301 are glioblastoma cells.

### 302 Neuroprotective Effect of *N*-Acetylcysteine, 303 Cystine, and Glutathione Ethyl Ester

304 Based on the viability assays, 15  $\mu\text{mol/L}$  Thimerosal  
 305 was selected as the dose for the supplementation  
 306 studies since a significant level of toxicity was  
 307 observed in both cell lines.

#### 308 Glioblastoma Cells

309 Triplicate cultures were pretreated for 1 h with  
 310 100  $\mu\text{mol/L}$  *N*-acetylcysteine, glutathione ethyl ester,  
 311 cystine, or methionine before addition of 15  $\mu\text{mol/L}$   
 312 Thimerosal to the culture medium. As shown in  
 313 Fig. 4A, 15  $\mu\text{mol/L}$  Thimerosal alone induced approxi-  
 314 mately 3-fold decrease in cell viability whereas pre-  
 315 treatment with either cystine, glutathione, or NAC  
 316 provided significant protection from cell death. The  
 317 failure of methionine to provide precursors for GSH  
 318 synthesis is consistent with the lack of cystathionine  $\gamma$   
 319 lyase activity in these cells.

#### 320 Neuroblastoma Cells

321 Triplicate cultures of neuroblastoma cells were pre-  
 322 treated for 1 h with the same supplements as the  
 323 glioblastoma cells. Fig. 4B demonstrates that Thimerosal  
 324 alone induced more than a 6-fold decrease in  
 325 viability, confirming the increased vulnerability of  
 326 neuroblastoma cells to Thimerosal relative to the gli-

327 blastoma cells. Both NAC and glutathione ethyl ester  
 328 provided significant protection against cell death  
 329 whereas cystine and methionine were without effect.  
 330 The lack of effect of cystine is consistent with previous  
 331 reports that neurons can take up cysteine, but not  
 332 cystine (Sagara et al., 1993). As expected, methionine  
 333 afforded no protection.

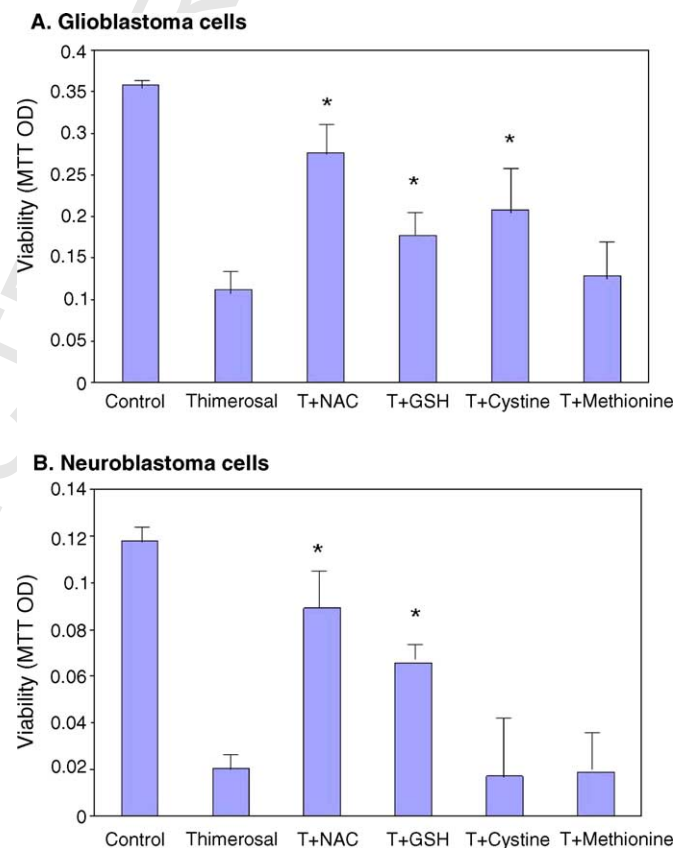


Fig. 4. Viability of glioblastoma cells (A) and neuroblastoma cells (B) without (control) and with exposure to 15  $\mu\text{mol/L}$  Thimerosal alone for 48 h or Thimerosal treatment after a 45 min pretreatment with 100  $\mu\text{mol/L}$  of *N*-acetyl cysteine (NAC), glutathione ethyl ester (GSH) cystine, or methionine. Asterisks indicate significant differences between cells treated with Thimerosal alone and cells pretreated with indicated nutrients ( $n = 3$ ,  $p < 0.05$ ).

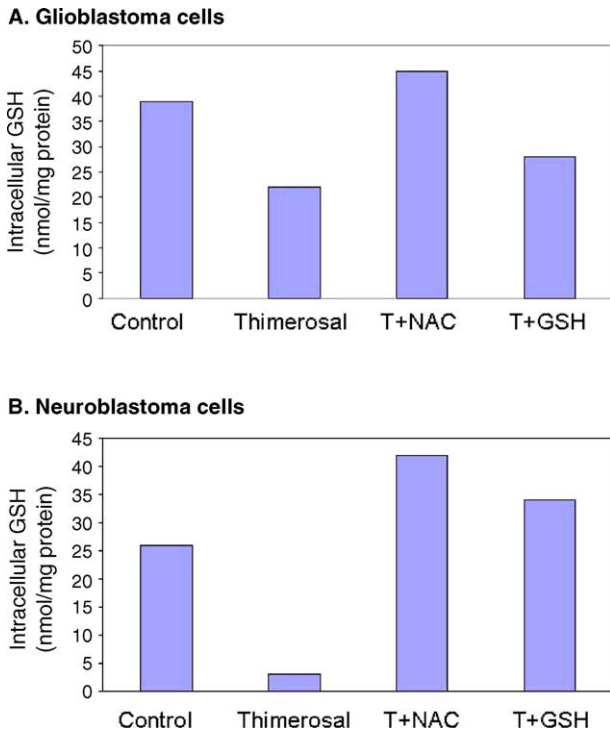


Fig. 5. Mean concentration of intracellular glutathione in 10<sup>6</sup> glioblastoma cells (A) and neuroblastoma cells (B) without (control) and with 15 μmol/L Thimerosal alone or Thimerosal after pretreatment with 100 μmol/L of N-acetyl cysteine (NAC), glutathione ethyl ester (GSE) in a representative experiment that was repeated with similar results.

**Glutathione Depletion with Thimerosal Exposure: Preservation of Intracellular GSH with Nutritional Supplementation**

The baseline level of intracellular glutathione was 39 μmol/L in the glioblastoma cells compared to only 26 μmol/L in the neuroblastoma cells (Fig. 5A). Exposure to 15 μmol/L Thimerosal for 1 h caused less than a 50% decrease in intracellular glutathione levels in the glioblastoma cells whereas the same exposure induced more than an 8-fold-decrease in the neuroblastoma cells. Pretreatment with NAC or glutathione ethyl ester completely prevented the Thimerosal-induced depletion in glutathione in neuroblastoma cells (Fig. 5B). In the glioblastoma cells, pretreatment with NAC completely prevented the Thimerosal-induced glutathione depletion and glutathione ethyl ester was partially protective.

**DISCUSSION**

Considerable concern has been expressed recently over the cumulative dose of mercury given to children through routine immunizations given in the 1990s. The

source of mercury in vaccines is the antimicrobial preservative, Thimerosal, containing 49.9% ethyl mercury by weight. All forms of mercury are well known to be neurotoxic, especially during early brain development (Costa et al., 2004). The high affinity binding of mercuric compounds to the thiol (–SH) group of cysteines in essential proteins is thought to be the basis for mercury-induced cytotoxicity. In vivo studies in rodents have shown that ethyl mercury is able to cross the cell membrane and then is converted intracellularly to inorganic mercury which accumulates preferentially in the brain and kidney (Magos et al., 1985). Intracellular accumulation of inorganic mercury was shown to be higher for ethyl compared to methylmercury with equimolar exposure, although the clearance rate of ethylmercury was faster than methylmercury (Magos et al., 1985). A recent in vitro study of Thimerosal exposure to fibroblasts and human cerebral cortical neuron cell lines demonstrated that short term exposure in concentrations similar to those used in the present study induced DNA strand breaks, membrane damage, caspase-3 activation, and cell death (Baskin et al., 2003). The purpose of the present study was to determine whether the mechanism of ethylmercury toxicity was similar to that previously reported for methylmercury. Human glioblastoma and neuroblastoma cell lines were used as surrogates for astrocytes and neurons, respectively, based on a previous study demonstrating similar dose–response profiles to methylmercury between primary cultures and transformed cell lines (Sanfeliu et al., 2001).

Glutathione provides the major intracellular defense against ROS and oxidative stress-induced cell damage and apoptosis (Meister, 1995). Agents or conditions that deplete mitochondrial glutathione will indirectly increase ROS levels and induce cell death in a variety of cell types (Allen et al., 2001; Marchetti et al., 1997). Mercury and other heavy metals are well known to increase oxidative stress and deplete intracellular glutathione (Naganuma et al., 1990). A major unanswered question is whether mercury-induced depletion of glutathione precedes the increase in ROS or whether mercury-induced ROS induces glutathione depletion. In thymocytes, mitochondrial glutathione depletion was shown to precede the increase in ROS associated with loss of viability and apoptosis (Macho et al., 1997). Whether mercury-induced depletion of glutathione is the initiating factor for increased oxidative stress and cell death in brain cells has not yet been evaluated.

A recent in vitro study of Thimerosal immunotoxicity using immortalized Jurkat T cells demonstrated an

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407 increase in reactive oxygen species and a decrease in  
 408 intracellular glutathione with increasing concentra-  
 409 tions of Thimerosal (Makani et al., 2002). Thimerosal,  
 410 but not thiosalicylic acid (the non-mercury component  
 411 of Thimerosal), induced apoptotic cell death in T cells  
 412 in a concentration-dependent manner as evidenced by  
 413 mitochondrial release of cytochrome *c*, apoptosis acti-  
 414 vating factor, and activation of caspases 9 and 3.  
 415 Exogenous glutathione inhibited activation of these  
 416 caspases and prevented cell death. These results sug-  
 417 gest that, at least in T cells, Thimerosal induces  
 418 oxidative stress and apoptosis by activating mitochon-  
 419 drial cell death pathways. A subsequent study using  
 420 cultured human neuron and fibroblast cell lines simi-  
 421 larly showed that low micromolar concentrations of  
 422 Thimerosal induced DNA strand breaks, caspase-3  
 423 activation, membrane damage and cell death (Baskin  
 424 et al., 2003).

425 In the present study, we evaluated glioblastoma  
 426 cells and neuroblastoma cells in culture to determine  
 427 the relative sensitivity of each cell type to Thimerosal-  
 428 induced oxidative stress and cell death. At equimolar  
 429 concentrations of Thimerosal, the neurons were found  
 430 to be much more sensitive to Thimerosal-induced cell  
 431 death than the astrocytes. In the neuronal cell line,  
 432 viability was significantly reduced in a concentration-  
 433 dependent manner at 2.5, 5, 10, and 20  $\mu\text{mol/L}$  Thi-  
 434 merosal after only a 3 h exposure, whereas the astro-  
 435 cytes required a full 48 h exposure for a similar loss of  
 436 viability (Fig. 3). These results duplicate observations  
 437 in the same cell lines exposed to similar concentra-  
 438 tions of methyl mercury and suggest that the mechan-  
 439 ism of ethyl- and methylmercury neurotoxicity is  
 440 similar. The addition of either *N*-acetylcysteine or  
 441 glutathione ethyl ester (100  $\mu\text{mol/L}$ ) to the culture  
 442 medium 45 min before adding 15  $\mu\text{mol/L}$  Thimerosal  
 443 conferred significant protection against cytotoxicity  
 444 in both cell lines (Fig. 4). It is likely that the extra-  
 445 cellular NAC and glutathione provided partial protec-  
 446 tion by complexing with the Thimerosal in the culture  
 447 medium as well as by increasing intracellular glu-  
 448 thathione levels. The oxidized form of cysteine  
 449 (cystine) was protective in astrocytes, but not neurons,  
 450 consistent with facilitated membrane transport of  
 451 cystine in astrocytes (Kranich et al., 1998). Neurons  
 452 depend on glutathione synthesized in the astrocytes  
 453 and released extracellularly where it is hydrolyzed to  
 454 cysteinylglycine and cysteine by ectoenzymes to pro-  
 455 vide neurons with necessary precursors for intracel-  
 456 lular glutathione synthesis (Dringen et al., 1999). In  
 457 both cell lines, methionine provided no protection  
 458 against Thimerosal toxicity confirming the inability

of either cell type to synthesize cysteine (and glu-  
 thathione) from methionine.

The intracellular concentration of glutathione before  
 supplementation was 30% lower in neuroblastoma  
 cells compared to the glioblastoma cells. The lower  
 baseline glutathione concentration in the neuronal cell  
 line was associated with increased sensitivity to Thi-  
 merosal cytotoxicity (Fig. 5). Thus, sensitivity to Thi-  
 merosal was directly proportional to the basal  
 intracellular glutathione concentration. In co-culture  
 studies, astrocytes have been shown to protect neurons  
 against the toxicity of oxidative stress (Dringen et al.,  
 2000a). The provision of glutathione precursors to  
 neurons is a possible explanation for the protective  
 effect of astrocytes. Recent results have confirmed the  
 primary role of astrocytes in glutathione metabolism  
 and antioxidant defense in the brain (Dringen, 2000).  
 Depletion of astrocyte glutathione would therefore  
 indirectly induce oxidative cell death in neurons by  
 depletion of essential glutathione precursors.

In summary, we have shown that human glioblas-  
 toma cells are more resistant to Thimerosal cytotoxi-  
 city than neuroblastoma cells at doses in the low  
 micromolar range and that the resistance is correlated  
 with higher intracellular levels of intracellular glu-  
 thathione. The significant protection by NAC and glu-  
 thathione ethyl ester against Thimerosal cytotoxicity  
 suggests the possibility that supplementation with glu-  
 thathione precursors might be protective against mer-  
 cury exposures in vivo. Numerous clinical studies have  
 demonstrated the efficacy of NAC in increasing intra-  
 cellular glutathione levels and reducing oxidative stress  
 in humans (Anderson and Luo, 1998; Badaloo et al.,  
 2002). Since cytotoxicity with both ethyl- and methyl-  
 mercury have been shown to be mediated by glu-  
 thathione depletion, dietary supplements that increase  
 intracellular glutathione could be envisioned as an  
 effective intervention to reduce previous or anticipated  
 exposure to mercury. This approach would be espe-  
 cially valuable in the elderly and in pregnant women  
 before receiving flu vaccinations, in pregnant women  
 receiving Rho D immunoglobulin shots, and indivi-  
 duals who regularly consume mercury-containing fish.

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