Children's
Health Defense

Peer-Reviewed, Published Research Showing Adverse Effects of Mercury

The literature showing the toxicity of mercury goes well beyond its associations with autism. This document includes the abstracts for over 240 studies that show the harmful effects of mercury, from both thimerosal and environmental sources, on brain cells, immune cells and other body systems. These include cellular, animal and human studies. There can be no justification for any intentional use of mercury given the extent of this literature.

The following pages are abstracts from the peer reviewed 240+ studies.

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Mercury, Lead, and Zinc in Baby Teeth of Children with Autism Versus Controls

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This study determined the level of mercury, lead, and zinc in baby teeth of children with autism spectrum disorder (n = 15, age 6.1 ± 2.2 yr) and typically developing children (n = 11, age = 7 ± 1.7 yr). Children with autism had significantly (2.1-fold) higher levels of mercury but similar levels of lead and similar levels of zinc. Children with autism also had significantly higher usage of oral antibiotics during their first 12 mo of life, and possibly higher usage of oral antibiotics during their first 36 mo of life. Baby teeth are a good measure of cumulative exposure to toxic metals during fetal development and early infancy, so this study suggests that children with autism had a higher body burden of mercury during fetal/infant development. Antibiotic use is known to almost completely inhibit excretion of mercury in rats due to alteration of gut flora. Thus, higher use of oral antiobiotics in the children with autism may have reduced their ability to excrete mercury, and hence may partially explain the higher level in baby teeth. Higher usage of oral antibiotics in infancy may also partially explain the high incidence of chronic gastrointestinal problems in individuals with autism.

Autism is a severe developmental disorder that involves social withdrawal, communication deficits, and stereotypic/repetitive behaviors. The causes of autism are unknown, but both genetic and environmental factors have been implicated. The purpose of this study was to investigate the environmental factor of heavy metals (mercury, lead) toxicity.

A thorough review by Bernard et al. (2001) reported that all of the major symptoms reported in the literature for autism were also reported for cases of infantile mercury poisoning, including especially language/communication problems and social withdrawal. Therefore, they suggested that autism was primarily due to infantile exposure to mercury. Their hypothesis is plausible because mercury exposure at hazardous levels is common in the United States and other countries; the Food and Drug Administration (FDA) estimates that 1 in 6 women in the United States have mercury levels that increase the risk of neurological damage to their children. (Mahaffey et al., 2004) The major sources of mercury exposure for infants are (1) maternal seafood consumption, (2) maternal mercury amalgam dental fillings, and (3) thimerosal (an ethylmercury compound) in childhood vaccines and in anti-RhoD immune globulins given to Rh-negative mothers during pregnancy. Thimerosal was largely but not totally removed from childhood vaccines by 2004.

Mercury toxicity might occur either due to high exposure, or due to a decreased ability to excrete mercury, with the latter case seeming to be the primary issue in autism. The primary mechanism for excreting mercury involves its binding to glutathione and then being excreted in the bile (Ballatori & Clarkson, 1985). Infants are poor excretors because they produce less glutathione (Ballatori & Clarkson, 1984) and because they are usually on all-milk diets (which decreased mercury excretion by a factor of 3 in a study of rats); thus, they are especially vulnerable to mercury poisoning (Rowland et al., 1984).

Infants with autism were even more vulnerable to mercury toxicity, because their glutathione is much lower than in typical children (James et al., 2004; Audhya, 2004) and a higher fraction of their glutathione is oxidized (James et al., 2004). Further, two studies (Konstantareas & Homatidis, 1987; Adams et al., 2003) found that children with autism had much higher usage of oral antibiotics, which (in rats) resulted in a near-total loss of the ability to excrete mercury (Rowland et al., 1980, 1984). The reason appears to be that normal gut anaerobes are able to convert methylmercury (which is rapidly absorbed) into inorganic mercury (which is poorly absorbed and hence mostly excreted). In contrast, most strains of yeast

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Research Article

The Severity of Autism Is Associated with Toxic Metal Body Burden and Red Blood Cell Glutathione Levels

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Recommended by Wei Zheng

This study investigated the relationship of children's autism symptoms with their toxic metal body burden and red blood cell (RBC) glutathione levels. In children ages 3–8 years, the severity of autism was assessed using four tools: ADOS, PDD-BI, ATEC, and SAS. Toxic metal body burden was assessed by measuring urinary excretion of toxic metals, both before and after oral dimercaptosuccinic acid (DMSA). Multiple positive correlations were found between the severity of autism and the urinary excretion of toxic metals. Variations in the severity of autism measurements could be explained, in part, by regression analyses of urinary excretion of toxic metals before and after DMSA and the level of RBC glutathione (adjusted R^2 of 0.22–0.45, $P < .005$ in all cases). This study demonstrates a significant positive association between the severity of autism and the relative body burden of toxic metals.

1. Background

Autism is a severe developmental disorder which involves social withdrawal, communication deficits, and stereotypic/repetitive behaviour. The pathophysiological etiologies which precipitate autism symptoms remain elusive and controversial in many cases, but both genetic and environmental factors (and their interactions) have been implicated. One environmental factor that has received significant attention is the body burden of mercury, lead, and other toxic metals [1–5].

Bernard et al. [1] discussed the many similarities between the symptoms of children with autism and children poisoned by mercury. An epidemiology study by Windham et al. [2] found that the amount of airborne pollutants, and especially mercury, correlated with an increased risk for autism. A study by DeSoto and Hitlan [3] found that blood levels of mercury did significantly correlate with the diagnosis of autism. A small study by Adams et al. [4] found that children with autism had a 2-time higher level of mercury in their baby teeth than typical children. A study by Bradstreet et al. [5] investigated the body burden of toxic metals by giving dimercaptosuccinic acid (DMSA), an oral chelation medication approved by the FDA for treating infantile lead poisoning. They found that the children with autism excreted 3.1 times as much mercury into their urine (which is where DMSA is excreted), $P < .0002$, but lead and cadmium levels were not significantly different. Overall there is some evidence to suggest that mercury and possibly other toxic metals are related to the etiology of autism.

This study investigates the possible relationship of the severity of autism to the relative body burden of toxic metals. The severity of autism was assessed using four tools, a professional evaluation based on the Autism Diagnostic Observation Schedule [6], and parental evaluations based on the Pervasive Developmental Disorders Behaviour Inventory

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Toxicological Status of Children with Autism vs. Neurotypical Children and the Association with Autism Severity

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Abstract This study investigates both the level of toxic metals in children with autism and the possible association of those toxic metals with autism severity. This study involved 55 children with autism ages 5–16 years compared to 44 controls with similar age and gender. The study included measurements of toxic metals in whole blood, red blood cells (RBC), and urine. The autism group had higher levels of lead in RBC (+41 %, p=0.002) and higher urinary levels of lead (+74 %, p=0.02), thallium (+77 %, p=0.0001), tin (+115 %, p=0.01), and tungsten (+44 %, p=0.00005). However, the autism group had slightly lower levels of cadmium in whole blood (−19 %, p=0.003). A stepwise, multiple linear regression analysis found a strong association of levels of toxic metals with variation in the degree of severity of autism for all the severity scales (adjusted R^2 of 0.38–0.47, p<0.0003). Cadmium (whole blood) and mercury (whole blood and RBC) were the most consistently significant variables. Overall, children with autism have higher average levels of several toxic metals, and levels of several toxic metals are strongly associated with variations in the severity of autism for all three of the autism severity scales investigated.

Keywords Autism · Toxic metals · Mercury · Lead · Thallium · Tungsten

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Background and Significance

Determination of toxic metal exposure in classic lead poisoning, such as due to ingestion of lead paint, is relatively easy and involves measuring blood levels of lead. However, in autism, the problem appears to usually not be high exposure, but rather decreased excretion. The half-life of lead, mercury, and other toxic metals in the blood is weeks to months, so those metals rapidly leave the blood and accumulate in tissue and/or bone. Since biopsies of those tissues are invasive, this makes assessment of toxic metal exposure in autism more complex.

Many studies suggest that children with autism have a decreased ability to excrete toxic metals, leading to a higher body burden. The decreased ability to excrete toxic metals is partly due to low glutathione [1–4] since glutathione conjugation (and subsequent excretion in the feces) is the primary pathway for removal of some toxic metals. Another factor that also decreases ability to excrete toxic metals in feces is increased use of oral antibiotics [5–8] since oral antibiotics have been shown (in rats) to almost completely inhibit excretion of mercury [9, 10] due to their effect on altering gut flora. This is consistent with two studies which found lower levels of mercury in the baby hair of children with autism, [8, 11], one study which found decreased lead,

PLOS ONE

RESEARCH ARTICLE

Significant Association of Urinary Toxic Metals and Autism-Related Symptoms—A Nonlinear Statistical Analysis with Cross Validation

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Abstract

Introduction

A number of previous studies examined a possible association of toxic metals and autism, and over half of those studies suggest that toxic metal levels are different in individuals with Autism Spectrum Disorders (ASD). Additionally, several studies found that those levels correlate with the severity of ASD.

Methods

In order to further investigate these points, this paper performs the most detailed statistical analysis to date of a data set in this field. First morning urine samples were collected from 67 children and adults with ASD and 50 neurotypical controls of similar age and gender. The samples were analyzed to determine the levels of 10 urinary toxic metals (UTM). Autism-related symptoms were assessed with eleven behavioral measures. Statistical analysis was used to distinguish participants on the ASD spectrum and neurotypical participants based upon the UTM data alone. The analysis also included examining the association of autism severity with toxic metal excretion data using linear and nonlinear analysis. "Leave-one-out" cross-validation was used to ensure statistical independence of results.

Results and Discussion

Average excretion levels of several toxic metals (lead, tin, thallium, antimony) were significantly higher in the ASD group. However, ASD classification using univariate statistics proved difficult due to large variability, but nonlinear multivariate statistical analysis significantly improved ASD classification with Type I/II errors of 15% and 18%, respectively. These results clearly indicate that the urinary toxic metal excretion profiles of participants in the ASD group were significantly different from those of the neurotypical participants. Similarly, nonlinear methods determined a significantly stronger association between the

Thimerosal induces TH2 responses via influencing cytokine secretion by human dendritic cells

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Abstract: Thimerosal is an organic mercury compound that is used as a preservative in vaccines and pharmaceutical products. Recent studies have shown a TH2-skewing effect of mercury, although the underlying mechanisms have not been identified. In this study, we investigated whether thimerosal can exercise a TH2-promoting effect through modulation of functions of dendritic cells (DC). Thimerosal, in a concentration-dependent manner, inhibited the secretion of LPS-induced proinflammatory cytokines TNF-α, IL-6, and IL-12p70 from human monocyte-derived DC. However, the secretion of IL-10 from DC was not affected. These thimerosal-exposed DC induced increased TH2 (IL-5 and IL-13) and decreased TH1 (IFN-γ) cytokine secretion from the T cells in the absence of additional thimerosal added to the coculture. Thimerosal exposure of DC led to the depletion of intracellular glutathione (GSH), and addition of exogenous GSH to DC abolished the TH2-promoting effect of thimerosal-treated DC, restoring secretion of TNF-α, IL-6, and IL-12p70 by DC and IFN-γ secretion by T cells. These data suggest that modulation of TH2 responses by mercury and thimerosal, in particular, is through depletion of GSH in DC. *J. Leukoc. Biol.* 81: 474–482; 2007.

Key Words: *APC · heavy metal · immune modulation*

INTRODUCTION

Exposure to mercury is widespread in the world, and inorganic mercury, ethylmercury, and methylmercury are the predominant chemical species. The primary sources of exposure to mercury are amalgam, mercury vapors, vaccination, and seafood consumption [1–3]. Thimerosal (ethylmercurithiosalicylate) is an organic mercury compound that has been used as a preservative in vaccines, intramuscular immune globulin preparations, skin test antigens, antivenoms, ophthalmic and nasal products, and tattoo inks [1–3]. It has 49.6% mercury by weight, and following its administration, its metabolite, ethylmercury, dissociates from thiosalicylic acid and binds to blood or other tissue. The extensive use of vaccines in today's society has led to concerns about immunization safety. Today, children receive more total number of vaccinations given together during the first two years of life, leading to exposure to quantities of mercury that exceeds the safety guidelines through thimerosal in vaccines. There is an increasing concern about association between the exposure to mercury (via vaccination) and the development of neurodevelopmental disorders, especially autism and learning disabilities [3–8]. This has led to thimerosal being withdrawn from pediatric vaccines in the United States starting in 1999 (Centers for Disease Control and Prevention, 1999). Nevertheless, thimerosal is still used in influenza, diphtheria toxoid and acellular pertussis, and tetanus toxoid vaccines. The majority of the studies are directed toward understanding the neurotoxic effect of thimerosal, and few studies deal with its effect on the immune system.

The effect of mercury on the immune system has been studied mostly in rodents. These studies have revealed that subtoxic doses of mercury exposure in genetically susceptible H-2 mice strains result in the development of systemic autoimmunity characterized by lymphoproliferation with polyclonal B cell activation and hyper-γ-globulinemia, production of autoantibodies targeting the 34-kDa nucleolar protein fibrillarin, and development of immune-complex deposits [9–15]. The different forms of mercury differ in the type and range of immune disorders, and ethylmercury (thimerosal) and inorganic mercury are similar in that they cause systemic autoimmunity, characterized by a marked increase of IgE and systemic immune-complex deposits [16, 17]. Antifibrillarin autoantibodies (AFA) and maximum levels of serum IgE are present as early as 10 days after exposure to ethylmercury in the mice [16]. Similar to the autoimmune disease induced by inorganic mercury, thimerosal induces a distinctly increased expression of IL-4 mRNA and a large increase in TH2-dependent, Ig-secreting cells and serum Igs [18]. The increase in IL-4 has been attributed to a direct induction of IL-4 gene expression in lymphocytes by mercury [19]. Methylmercury, conversely, induces only modest titers of AFA and none of the above symptoms [16, 17, 20]. One of the possible explanations is that ethylmercury is converted much faster into inorganic mercury compared with methylmercury, leading to an earlier and more potent effect on the immune system. The immunosuppressive effects of ethyl and methyl mercury are similar and more potent than inorganic mercury in that they both cause reduction in the number and proliferative capacity of splenic T and B lymphocytes [17, 20]. Studies in humans document

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Review

Genetic Aspects of Susceptibility to Mercury Toxicity: An Overview

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Abstract: Human exposure to mercury is still a major public health concern. In this context, children have a higher susceptibility to adverse neurological mercury effects, compared to adults with similar exposures. Moreover, there exists a marked variability of personal response to detrimental mercury action, in particular among population groups with significant mercury exposure. New scientific evidence on genetic backgrounds has raised the issue of whether candidate susceptibility genes can make certain individuals more or less vulnerable to mercury toxicity. In this review, the aim is to evaluate a new genetic dimension and its involvement in mercury risk assessment, focusing on the important role played by relevant polymorphisms, located in attractive gene targets for mercury toxicity. Existing original articles on epidemiologic research which report a direct link between the genetic basis of personal vulnerability and different mercury repercussions on human health will be reviewed. Based on this evidence, a careful evaluation of the significant markers of susceptibility will be suggested, in order to obtain a powerful positive "feedback" to improve the quality of life. Large consortia of studies with clear phenotypic assessments will help clarify the "window of susceptibility" in the human health risks due to mercury exposure.

Keywords: mercury; toxicokinetics; human health; risk assessment; children exposure; environmental genetics; DNA variants; biomarkers of susceptibility

1. Introduction

Mercury (Hg) is a global pollutant and well-known neurotoxin that has raised great fear in the international scientific community, due to a variety of significant and documented adverse effects on human health and the environment throughout the world [1]. Despite being a well-documented systemic toxicant, an understanding of all the molecular mechanisms underlying the damage induced by Hg is still elusive.

The need to further reduce Hg emissions, as well as to develop preventive strategies in relation to Hg risk assessment and management makes the situation even more challenging, especially for those individuals most susceptible to the effects of Hg exposure, such as children and adolescents [1]. The two categories are highly sensitive to the neurotoxic Hg effects, displaying extreme variability in mainly neurological and neurobehavioural outcomes throughout subsequent life stages [2].

The pathological impact of Hg on humans and other organisms is widely proven, and the overall picture is quite complex. Human exposure may occur chronically through a variety of pathways in the world population, including industrial processes, occupational and household uses, dental amalgams, Hg-containing vaccines, consumption of contaminated fish and marine mammals, and many others [2]. To date, two main types of risk for human health have been detected: a direct one, related to the inhalation of gaseous Hg, with several pathophysiological impacts, and collateral risks, related to differences between Hg species.

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An Investigation of Porphyrinuria in Australian Children with Autism

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Two recent studies, from France (Nataf et al., 2006) and the United States (Geier & Geier, 2007), identified atypical urinary porphyrin profiles in children with an autism spectrum disorder (ASD). These profiles serve as an indirect measure of environmental toxicity generally, and mercury (Hg) toxicity specifically, with the latter being a variable proposed as a causal mechanism of ASD (Bernard et al., 2001; Mutter et al., 2005). To examine whether this phenomenon occurred in a sample of Australian children with ASD, an analysis of urinary porphyrin profiles was conducted. A consistent trend in abnormal porphyrin levels was evidenced when data was compared with those previously reported in the literature. The results are suggestive of environmental toxic exposure impairing heme synthesis. Three independent studies from three continents have now demonstrated that porphyrinuria is concomitant with ASD, and that Hg may be a likely xenobiotic to produce porphyrin profiles of this nature.

Autism is a neurodevelopmental disorder presenting in childhood that affects up to 1 in 150 children in the United States (Centers for Disease Control, 2006) and 1 in 160 in Australia (Wray & Williams, 2007). Autism is characterized by severe impairments in socialization, communication, and behavior (American Psychiatric Association, 1994). The prevalence of autism is increasing at epidemic rates (Yazbak, 2003) that cannot be accounted for by changing diagnostic criteria or improved diagnostic systems (Blaxill et al., 2003; Croen et al., 2002).

Mercury (Hg) toxicity has been proposed as a causal mechanism whereby a small subset of children are uniquely sensitive to Hg and, in such individuals, exposure triggers a cascade of events leading to autism (Bernard et al., 2001; Mutter et al., 2005; Kern & Jones, 2006). Urinary porphyrins provide a convenient and non-invasive measure of xenobiotic exposure generally (Brewster, 1988) and of Hg specifically (Woods et al., 2005; Heyer et al., 2006).

Excess urinary porphyrin excretion (porphyrinuria) results from the inhibition of enzymatic steps in conditions including genetic deficiencies in heme production enzymes, hepatitis, renal disease, and erythroid disease (Gross et al., 2000), as well as by heavy metal inhibition (Bowers et al., 1992; Woods, 1996). The causal relationship between Hg and porphyrinuria has been demonstrated both in rats (Pingree et al., 2001) and in humans (Woods et al., 1993).

The steps in the heme pathway most vulnerable to heavy metal inhibition are those that involve uroporphyrin decarboxylase (Woods & Kardish, 1983) and coproporphyrinogen oxidase (Woods et al., 2005). The result of these inhibitions is specific elevations of urinary coproporphyrin and pentacarboxyporphyrin levels. Although nonmetal agents targeting the heme pathway also elevate urinary porphyrin levels (Daniell et al., 1997), precoproporphyrin (also known as keto-isocoproporphyrin) is produced by in vivo conversion of pentacarboxyporphyrin in the presence of heavy metal, providing a specific porphyrin marker for Hg exposure (Woods et al., 2005; Heyer et al., 2006).

Two previous studies reported porphyrinuria among autistic subjects consistent with elevated body burden of Hg (Nataf et al., 2006; Geier & Geier, 2007). The pattern is one of generalized porphyrinuria with marked elevation of coproporphyrin and of precoproporphyrin and of the ratio of coproporphyrin/uroporphyrin. This study aimed to examine this phenomenon among a group of Australian autistic children.

METHODS

Subjects

Urinary porphyrin profiles were obtained from 41 consecutive patients with an ASD presenting to the first author's psychology clinic from October 2006 through March 2008. Each patient was previously diagnosed with an ASD, by a health professional, based upon accepted international

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Thimerosal Exposure in Early Life and Neuropsychological Outcomes 7–10 Years Later

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Objective The authors used a public use data set to investigate associations between the receipt of thimerosal-containing vaccines and immune globulins early in life and neuropsychological outcomes assessed at 7–10 years. **Methods** The data were originally created by evaluating 1,047 children ages 7–10 years and their biological mothers. This study developed seven latent neuropsychological factors and regressed them on a comprehensive set of covariates and thimerosal exposure variables. **Results** The authors found no statistically significant associations between thimerosal exposure from vaccines early in life and six of the seven latent constructs. There was a small, but statistically significant association between early thimerosal exposure and the presence of tics in boys. **Conclusions** This finding should be interpreted with caution due to limitations in the measurement of tics and the limited biological plausibility regarding a causal relationship.

Key words children; modeling; neuropsychology; public health; structural equation.

Introduction

The association between exposure to thimerosal-containing vaccines and developmental outcomes has been debated since 1999 (Bernard, 2008; Clements, Ball, Ball, & Pratt, 2001; Offit, 2007; Rooney, 2008; Sugarman, 2007) when the Food and Drug Administration (FDA) determined that children who received multiple thimerosal containing vaccines at a young age were at risk for exceeding the Environmental Protection Agency's (EPA) safety limits for methylmercury (Ball, Ball, & Pratt, 2001; Stratton, Gable, & McCormick, 2001). EPA had never determined safety limits for ethylmercuty, the compound found in thimerosal containing vaccines. EPAs methylmercury saftey limits had been determined based on previous studies that found associations between methylmercury exposure and neuropsychological outcomes (Crump, Kjellstrom, Shipp, Silvers, & Stewart, 1998; Grandjean et al., 1999). Specifically, the Faroe Island studies found that high levels of methylmercury exposure due to maternal consumption of mercury-contaminated fish during pregnancy have been associated with children exhibiting lower motor function and verbal skills at 7 years of age (Grandjean et al., 1999). As a precautionary measure, the U.S. Public Health Service recommended the removal of thimerosal from vaccines administered to children early in life and the Centers for Disease Control and Prevention (CDC) proceeded to sponsor several studies investigating the possible associations between exposure to thimerosal-containing vaccines

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Thimerosal Induces DNA Breaks, Caspase-3 Activation, Membrane Damage, and Cell Death in Cultured Human Neurons and Fibroblasts

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Thimerosal is an organic mercurial compound used as a preservative in biomedical preparations. Little is known about the reactions of human neuronal and skin cells to its micro- and nanomolar concentrations, which can occur after using thimerosal-containing products. A useful combination of fluorescent techniques for the assessment of thimerosal toxicity is introduced. Short-term thimerosal toxicity was investigated in cultured human cerebral cortical neurons and in normal human fibroblasts. Cells were incubated with 125-nM to 250-μM concentrations of thimerosal for 45 min to 24 h. A 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) dye exclusion test was used to identify nonviable cells and terminal transferase-based nick-end labeling (TUNEL) to label DNA damage. Detection of active caspase-3 was performed in live cell cultures using a cell-permeable fluorescent caspase inhibitor. The morphology of fluorescently labeled nuclei was analyzed. After 6 h of incubation, the thimerosal toxicity was observed at 2 μM based on the manual detection of the fluorescent attached cells and at a 1-μM level with the more sensitive GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence. The lower limit did not change after 24 h of incubation. Cortical neurons demonstrated higher sensitivity to thimerosal compared to fibroblasts. The first sign of toxicity was an increase in membrane permeability to DAPI after 2 h of incubation with 250 μM thimerosal. A 6-h incubation resulted in failure to exclude DAPI, generation of DNA breaks, caspase-3 activation, and development of morphological signs of apoptosis. We demonstrate that thimerosal in micromolar concentrations rapidly induce membrane and DNA damage and initiate caspase-3–dependent apoptosis in human neurons and fibroblasts. We conclude that a proposed combination of fluorescent techniques can be useful in analyzing the toxicity of thimerosal.

Key Words: **thimerosal; active caspase-3; apoptosis; toxicity; neurons; fibroblasts; DNA breaks; membrane damage; DAPI.**

Thimerosal (sodium ethylmercury-thiosalicylate) is an antibacterial and antifungal mercurial compound used as a preservative in biological products and vaccines, in concentrations ranging from 0.003 to 0.01% (30–100 μg/ml) (Ball *et al*., 2001). Thimerosal contains 49.6 % mercury by weight and releases ethylmercury as a metabolite. In the body, ethylmercury can be converted to inorganic mercury, which then preferentially accumulates in the kidneys and brain (Blair *et al.*, 1975). Inorganic mercury is known to induce membrane and DNA damage (Ferrat *et al*., 2002; Ben-Ozer *et al*., 2000), and in cell culture conditions it was shown to be mutagenic and generate DNA breaks in concentrations below 500 nM (Schurz *et al*., 2000). Ethylmercury can significantly increase the concentration of inorganic mercury in many organs (Magos *et al*., 1985). After *in vivo* administration, ethylmercury passes through cellular membranes and concentrates in cells in vital organs, including the brain, where it releases inorganic mercury, raising its concentrations higher than equimolar doses of its close and highly toxic relative methylmercury (Magos *et al*., 1985).

However, little is known about acute reactions of various types of human cells following short-time exposure to thimerosal in micro- and nanomolar concentrations.

In this paper we used a convenient and easily reproducible combination of fluorescent techniques analyzing various markers of DNA and membrane damage, and investigated the toxicity of micromolar and nanomolar concentrations of thimerosal (125 nM–250 μM) occurring in the first 24 h of exposure in cultures of human cortical neuronal cells and in human fibroblasts.

We found that thimerosal in micromolar concentrations rapidly decreased cellular viability. Within several h after thimerosal administration, cells lost their capability to exclude the fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and developed multiple DNA breaks accompanied by caspase-3 activation and apoptotic morphology. Neuronal cell cultures demonstrated a higher sensitivity to thimerosal compared with fibroblasts.

MATERIALS AND METHODS

Cell cultures. HCN-1A Human cerebral cortical neurons (CRL-10442) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to ATCC recommendations. The line was

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Autism: a novel form of mercury poisoning

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Summary Autism is a syndrome characterized by impairments in social relatedness and communication, repetitive behaviors, abnormal movements, and sensory dysfunction. Recent epidemiological studies suggest that autism may affect 1 in 150 US children. Exposure to mercury can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with autism, and the similarities extend to neuroanatomy, neurotransmitters, and biochemistry. Thimerosal, a preservative added to many vaccines, has become a major source of mercury in children who, within their first two years, may have received a quantity of mercury that exceeds safety guidelines. A review of medical literature and US government data suggests that: (i) many cases of idiopathic autism are induced by early mercury exposure from thimerosal; (ii) this type of autism represents an unrecognized mercurial syndrome; and (iii) genetic and non-genetic factors establish a predisposition whereby thimerosal's adverse effects occur only in some children. © 2001 Harcourt Publishers Ltd

INTRODUCTION

Austic spectrum disorder (ASD) is a neurodevelopmental syndrome with onset prior to age 36 months. Diagnostic criteria consist of impairments in sociality and communication plus repetitive and stereotypic behaviors (1). Traits strongly associated with autism include movement disorders and sensory dysfunctions (2). Although autism may be apparent soon after birth, most autistic children experience at least several months, even a year or more of normal development – followed by regression, defined as loss of function or failure to progress (2–4).

The neurotoxicity of mercury (Hg) has long been recognized (5). Primary data derive from victims of contaminated fish (Japan – Minamata disease) or grain (Iraq, Guatemala, Russia); from acrodynia (Pink disease) induced by Hg in teething powders; and from individual instances of mercury poisoning (HgP), many occurring in occupational settings (e.g. Mad Hatter's disease). Animal and in vitro studies also provide insights into the mechanisms of Hg toxicity. More recently, the Food and Drug Administration (FDA) and the American Academy of Pediatrics (AAP) have determined that the typical amount of Hg injected into infants and toddlers via childhood immunizations has exceeded government safety guidelines on an individual (6) and cumulative vaccine basis (7). The mercury in vaccines derives from thimerosal (TMS), a preservative which is 49.6% ethylmercury (eHg) (7).

Past cases of HgP have presented with much inter-individual variation, depending on the dose, type of mercury, method of administration, duration of exposure, and individual sensitivity. Thus, while commonalities exist across the various instances of HgP, each set of variables has given rise to a different disease manifestation (8–11). It is hypothesized that the regressive form of autism represents another form of mercury poisoning, based on a thorough correspondence between autistic and HgP traits and physiological abnormalities, as well as on the known exposure to mercury through vaccines. Furthermore, other phenomena are consistent with a causal Hg-ASD relationship. These include: (a) symptom onset shortly after immunization; (b) ASD prevalence increases corresponding to vaccination increases; (c) similar sex ratios of affected individuals; (d) a high heritability rate for autism paralleling a genetic predisposition to

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TOXICOLOGY OF NEURODEVELOPMENTAL DISORDERS

The role of mercury in the pathogenesis of autism

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder of unknown etiology in most cases. Studies of monozygotic twins report an average 60% concordance rate, indicating a role for both genetic and environmental factors in disease expression.[1] Recent reviews in environmental health have suggested that early exposure to hazardous substances may underlie some cases of neurodevelopmental disorders, including ADHD, learning disabilities, and speech/language difficulties.[2] In 1999, thimerosal used as a vaccine preservative was identified as a widespread source of organic mercury exposure in infants.[3] Mercury (Hg), a heavy metal, is considered highly neurotoxic.[4] The amount of mercury in vaccines, while small, exceeded USEPA safety guidelines on a cumulative basis.[3] Certain individuals may exhibit severe adverse reactions to low doses of Hg which are otherwise largely benign to the majority of those exposed.[5] Some individuals with idiopathic autism spectrum disorder may represent such a sensitive population. As summarized in this paper, disease characteristics suggest this possibility: (a) ASD traits are known to arise from mercury exposure; (b) onset of ASD symptoms is temporally associated with administration of immunizations; (c) the reported increase in the prevalence of autism in the 1990s closely follows the introduction of two mercury-containing vaccines; and (d) elevated mercury has been detected in biological samples of autistic patients. Since ASD may now affect as many as one in 150 US children,[6] and since thimerosal is still used in many products worldwide, confirmation of thimerosal as an environmental agent in autism pathogenesis has important societal and patient implications.

Thimerosal is comprised of 49.6% ethylmercury (EtHg) by weight. Until early 2001, it was a component of most Hepatitis B, *Haemophilus influenzae* type B (HiB), and Diphtheria/Tetanus/Pertussis (DTP or DTaP) vaccines. These vaccines were routinely administered to infants at birth and at ages 2, 4, 6, and 15–18 months. The cumulative amount of mercury injected in the first 6 months of life was 187.5 μg.[3] Although the pharmacokinetics of EtHg have not been well studied, its toxicity is believed to be similar to MeHg,[3] for which pharmacokinetic models have been developed to estimate the risk of adverse outcomes based on Hg levels in standard biomarkers of hair or blood. Using such a model, the EtHg from the recommended vaccines is predicted to raise hair mercury levels above USEPA guidelines of 1 ppm for up to one year and, in some infants, to elevate Hg levels to 10 ppm, which is the lowest threshold for adverse outcomes in children exposed prenatally to MeHg.[4,7] That thimerosal-containing vaccines can significantly raise blood Hg levels in infants has been demonstrated *in vivo*.[8] Endpoints for adverse effects at low doses of MeHg have been in domains characteristic of ASD and include lowered performance on tests of attention, memory, language, and fine motor skills.[9–11] A CDC analysis of computerized HMO medical records found statistically significant associations between increased exposure to thimerosal from infant immunizations and attention deficit disorder, speech/language delay, and tics.[12] Traits characteristic of these disorders are common features of ASD.[10,11]

A review of medical literature has shown that exposure to mercury, whether organic or inorganic, can give rise to the symptoms and traits defining or commonly found in ASD individuals.[13] Mercury can cause impairments in social interaction, communication difficulties, and repetitive and stereotyped patterns of behavior, which comprise the three DSM-IV autism diagnostic criteria. Additionally, mercury can induce features prominent in ASD such as sensory abnormalities, emotional/psychological changes, movement disorder, impairments in abstract or complex thinking, severe sleep disturbances, and self injurious behavior. Males are more affected than females in both conditions. Physiological abnormalities more common in ASD populations and known to be caused by mercury exposure include gastrointestinal problems, autonomic nervous system disturbance, unusual EEG activity, immune system alterations, irregularities in neurotransmitter systems, and non-specific brain lesions.

The discovery and increase in the reported prevalence of autism parallels the introduction and spread of thimerosal-containing vaccines. Autism was first described in 1943 among children born in the 1930s.[14] Thimerosal was first added to childhood vaccines in the 1930s.[3] Prior to 1970, classic autism was estimated to occur in approximately 1 in 2000 children, while the average prevalence reported by studies from 1970 to 1990 is 1 in 1000.[15] This period was a time of increased immunization in the developed world. By 1995, the National Institutes of Health reported an autism prevalence of 1 in 500 children,[16] and in 2000 the CDC identified approximately 1 in 250 children with classic autism in one New Jersey town.[6] It was in the early 1990s that the thimerosal-containing HiB and Hepatitis B vaccines became part of the routine infant schedule.[3]

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Association Between Thimerosal-Containing Vaccine and Autism

To the Editor: In their article on the association between thimerosal-containing vaccines and autism, Dr Hviid and colleagues[1] acknowledged their affiliations with Statens Serum Institut, Copenhagen, Denmark, but did not disclose that the institute is a for-profit, state-owned enterprise with roughly $120 million in annual revenue. According to its 2002 Annual Report,[2] vaccines represent approximately one half of Statens Serum Institut's revenues and more than 80% of its profits. Furthermore, Statens Serum Institut manufactured the now discontinued monocomponent pertussis vaccine that contained thimerosal under investigation in their study. They were also the providers of diphtheria and tetanus components of a major thimerosal-containing diphtheria and tetanus toxoids and acellular pertussis vaccine (DTaP) vaccine sold in the United States.[3]

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To the Editor: Dr Hviid and colleagues[1] found no increase in relative risk of core autism from thimerosal in vaccines using the Danish autism registry. Denmark removed thimerosal from infant vaccines in mid-1992. The findings of Hviid et al are based on finding fewer older (born 1990-1992) thimerosal-exposed children than younger (born 1992-1996) unexposed children in the 2000 registry year. However, a sizable percentage of autism cases, skewing toward older children, are lost from the registry each year. Thus, the authors' finding is likely to be biased due to incomplete recordkeeping.

For instance, the 1995 registry[2] contains 97 cases among 5- to 9-year-olds. This same cohort, as it grows older, becomes the 10- to 14-year-old cohort in the 2000 registry, where its number has decreased to 75 children, a decline of 22 cases or 23% of the original 1995 group. Hviid et al stated that virtually all cases in their autism group were accurately diagnosed, and thus it is unlikely that cases were removed due to subsequent discovery of misdiagnosis and reclassification. Autism is a lifelong disorder with near-normal lifespan,[3] and few registry cases are in older age groups likely to die. Therefore, virtually any case entered into the registry should remain there. That some do not suggests administrative error.

I calculated the extent of record loss for the 1991-2000 span studied by Hviid et al. For each year, I added the number of newly enrolled cases for that year to the number of previous year's cases. I compared this total to the number of cases actually recorded in the registry for that year. For 4 of the years, the proportion lost amounts to one fourth of the cases. For the 2000 registry year, 23% of the cases from the previous year are missing. Cumulatively, 815 cases were dropped between 1991 and 2000, more than the total number remaining in 2000.

Removed cases accumulate each year, so for any given registry year, proportionately more removed cases fall into older age groups, because with each successive year, the removed cases get older. The effect is a bias toward more accurate counting of younger age cohorts while undercounting older ones. The relative risk and conclusions of Hviid et al are predicated on finding fewer cases in the older thimerosal cohort and more in the younger nonthimerosal groups. This is an untenable approach given the recordkeeping problem, and thus Hviid et al should either adjust their 2000 data for record loss or use an alternative methodology.

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In Reply: In response to Dr Rimland, the Statens Serum Institut is the national center for prevention and control of infectious diseases in Denmark. It is a nonprofit state enterprise under the auspices of the Danish Ministry of Health and Interiors. Thus, any profit belongs to the state.

GUIDELINES FOR LETTERS. Letters discussing a recent *JAMA* article will have the best chance of acceptance if they are received within 4 weeks of the article's publication. They should not exceed 400 words of text and 5 references. Letters reporting original research should not exceed 600 words and 6 references. All letters should include a word count. Letters must not duplicate other material published or submitted for publication. Letters will be published at the discretion of the editors and are subject to editing and abridgment. A signed statement for authorship criteria and responsibility, financial disclosure, copyright transfer, and acknowledgment is required for publication. Letters not meeting these specifications are generally not considered. Letters will not be returned unless specifically requested. Also see Instructions for Authors (July 2, 2003). We prefer that letters be submitted electronically to jama-letters@jama-archives.org. Letters may also be sent by surface mail to Letters Editor, *JAMA*, 515 N State St, Chicago, IL 60610, or by fax to (312) 464-5225 (please also send a hard copy via surface mail).

Letters Section Editor: Stephen J. Lurie, MD, PhD, Senior Editor.

treated with oral melphalan plus high-dose oral dexamethasone, the 3-year overall survival rate was 80%, showing that they were actually "good risk" patients. With censoring of data for patients who died early and patients who could not receive their assigned treatment, the results of the landmark analysis strongly argued against the superiority of high-dose melphalan, even in groups with 0% treatment-related mortality and 100% treatment feasibility. This probably resulted from the very similar hematologic response rates in the two treatment groups, in a disease in which a clonal response is mandatory for improved survival.

Our 24% rate of treatment-related mortality with high-dose melphalan is in keeping with the results of several other multicenter studies and can be considered as representative of the results with high-dose melphalan when used outside some tertiary referral centers. The better results obtained in these referral centers probably reflect not only better management of the disease but also better selection of candidates for high-dose melphalan. Both were likely factors in the impressive results reported by Comenzo et al. Studies comparing new standard-dose regimens with (optimized) high-dose treatments should now be performed in tertiary referral centers. In our opinion, further improvements in the survival of patients with AL amyloidosis are likely to result from the use of new drugs and innovative therapeutic approaches.

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Early Thimerosal Exposure and Neuropsychological Outcomes

TO THE EDITOR: Thompson et al. (Sept. 27 issue)[1] report the results of a study investigating the neuropsychological outcomes of early exposure to thimerosal. As a dissenting member of the panel of external consultants for this study, I object to the authors' conclusion that there is no causal association between thimerosal and children's brain function. The sample comprised children who were least likely to exhibit neuropsychological impairments. Specifically, children with congenital problems, those from multiple births, those of low birth weight, and those not living with their biological mother were excluded. The sample was skewed toward higher socioeconomic status and maternal education — factors that are associated with lower rates of neurobehavioral problems and higher intervention rates and that were not measured. The sampling frame included only children enrolled from birth in the health maintenance organization (HMO) and still enrolled after 7 to 10 years, excluding children in higher-mobility families, who tend to have lower academic and behavioral function.[2] Children with neurobehavioral problems may have been less likely to remain with the HMO. Only 30% of families selected for recruitment participated, a low rate for scientific research. Among the families selected for recruitment, 26% refused to participate. Another 28% "could not be located," which included families that did not respond to multiple recruitment attempts (internal documentation from the study contractor, Abt Associates) — another form of refusal.

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TO THE EDITOR: Recently, I summarized several nutritional factors that are likely to play a large

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The value of ecologic studies: mercury concentration in ambient air and the risk of autism

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Abstract

Ecologic studies of the spatial relationship between disease and sources of environmental contamination can help to ascertain the degree of risk to populations from contamination and to inform legislation to ameliorate the risk. Population risks associated with persistent low-level mercury exposure have recently begun to be of concern and current reports implicate environmental mercury as a potential contributor in the etiology of various developmental and neurodegenerative diseases including autism and Alzheimer's disease. In this demonstration of preliminary findings, we demonstrate for Bexar County Texas and Santa Clara County California, the hypothesis that the spatial structure of the occurrence of autism has a positive co-variation with the spatial structure of the distribution of mercury in ambient air. The relative risk of autism is greater in the geographic areas of higher levels of ambient mercury. We find that the higher levels of ambient mercury are geographically associated with point sources of mercury emission, such as coal-fired power plants and cement plants with coal-fired kilns. Although this does not indicate a cause, these results should not be dismissed, but rather seen as a preliminary step for generating a hypothesis for further investigation.

Keywords: ambient mercury; autism; ecologic analysis; point sources.

Introduction

Ecologic designs can serve as preliminary diagnostics of population health. A major aim in studying the geographic variation in health outcomes in ecologic designs is to formulate hypotheses about the etiology of disease by taking into account the spatial co-variation between the disease outcome and environmental factors. Furthermore, the visualization and exploration of co-varying spatial structures can lead to specifying statistical models that explain why one structure varies in response to another (1). Palmer et al. (2) demonstrated a significant association between increased environmental mercury release at the county level and increased rates of autism at the level of the school district across Texas. Ming et al. (3) found concentrations of autism spectrum disorders (ASDs) geographically associated with toxic landfills across the United States (US). Tang et al. (4) demonstrated that exposure to pollutants from a coal-fired power plant in a province of China adversely affected the development of children living in the area. In their 2009 article, Palmer et al. (5) also demonstrated a significant positive association between ambient mercury emission sources and rates of autism across Texas, using geographic proximity to the pollution source as a predictor variable. The results of this study suggested that distance to the source of environmental release explains the association between pounds of release and autism rates. These findings are consistent with prior literature demonstrating that proximity to mercury sources is related to greater loads of mercury in soil and plants (6, 7) and in human studies of occupational exposure (8, 9). Although their study was not intended to link cases of autism to sources of mercury contamination, Van Meter et al. (10) identified geographic clusters of cases of autism in California as a 'first hypothesis-generating step aimed at localized environmental exposures'.

In an unpublished analysis, we used data from the US Environmental Protection Agency (US EPA) National Air Toxics Assessment of 1996 (NATA, available from: http://www.epa.gov/ttn/atw/nata/), which is based on a comprehensive analysis of mercury emissions obtained from various State and local air pollution control agencies and from existing databases related to the air toxics regulatory program of the US EPA, including the Toxic Release Inventory. Ambient air mercury compound concentration density estimates were reported in tons per year, per square mile for each county in the US. Using statewide level autism data from the US Department of Education Office of Special Education and Rehabilitative Services 25th report to congress, and statewide estimates of the 1996 NATA data, we show that autism rates among children aged 3–5 years old in 2000 (namely, children conceived or born between 1995 and 1997 – the period during which the 1996 mercury emissions assessment was performed) were significantly higher among states having higher concentrations of ambient air mercury per square mile. Figure 1 depicts this association. The association remains

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Research Article

Mercury Disposition in Suckling Rats: Comparative Assessment Following Parenteral Exposure to Thiomersal and Mercuric Chloride

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Due to the facts that thiomersal-containing vaccine is still in use in many developing countries, and all forms of mercury have recognised neurotoxic, nephrotoxic, and other toxic effects, studies on disposition of ethylmercury and other mercury forms are still justified, especially at young age. Our investigation aimed at comparing mercury distribution and rate of excretion in the early period of life following exposure to either thiomersal (TM) or mercuric chloride ($HgCl_2$) in suckling rats. Three experimental groups were studied: control, TM, and $HgCl_2$, with 12 to18 pups in each. Both forms of mercury were administered subcutaneously in equimolar quantities (0.81 μmol/kg b.w.) three times during the suckling period (on the days of birth 7, 9, and 11) to mimic the vaccination regimen in infants. After the last administration of TM or $HgCl_2$, total mercury retention and excretion was assessed during following six days. In TM-exposed group mercury retention was higher in the brain, enteral excretion was similar, and urinary excretion was much lower compared to $HgCl_2$-exposed sucklings. More research is still needed to elucidate all aspects of toxicokinetics and most harmful neurotoxic potential of various forms of mercury, especially in the earliest period of life.

1. Introduction

Mercury is a pervasive environmental contaminant with proven toxic properties in mammals. Major risks recognized due to mercury exposure are dietary methylmercury exposure from fish and seafood, elemental mercury vapour from amalgam in tooth "silver fillings," and thiomersal-contained ethylmercury in vaccines [1–3]. Thiomersal (thimerosal, merthiolate) has been banned in the United States and Canada since 1999 and in the European Union since 2001 from vaccines recommended for children below seven years [4–6].

The molecule of thiomersal is sodium ethylmercury-thiosalycilate that dissociates to ethylmercury and thiosalicylate [7]. Ethylmercury is acting as a preservative against bacterial and fungal contamination of the vaccines that are repeatedly given to infants (Diphtheria-Tetanus-acellular-Pertussis vaccine, 3 to 7 times) up to 6 months of age. A potential threat of neurodevelopmental toxic effect of mercury lies in the fact that the exposure occurs in the most vulnerable period of life, when the brain is developing and growing [8]. Organic forms of mercury are more easily absorbed when ingested and are less readily eliminated from the body than its inorganic forms [1].

By now considerable amount of evidence has been collected to prove that doses of thiomersal in human vaccines do not pose harm, except for the risk of local hypersensitivity reactions [9–19]. In a recent overview Dórea [20] integrated experimental neurotoxicity studies of low-dose thiomersal in vaccines and concluded that doses relevant to thiomersal-containing vaccines exposure possess the potential to affect human neurodevelopment. A recently published experimental study in thiomersal-exposed infant rats reopens the debate on thiomersal-induced neurotoxic threat showing perturbations in the balance between excitatory and inhibitory amino acids in the brain, shifting it towards excessive neuroexcitation that may lead to neurodevelopmental disorders [21].

MÆDICA - a Journal of Clinical Medicine

ORIGINAL PAPERS

Efficacy of DMSA Therapy in a Sample of Arab Children with Autistic Spectrum Disorder

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ABSTRACT

Objective: *the aim of this study was to provide evidence that DMSA detoxification treatments cause a reduction of the heavy metal burden in the autistic, and that this reduction lessens neurological symptoms associated with ASD (Autistic Spectrum Disorder).*

Method: *The participants were 44 children, age 3 to 9 years of age, with Autistic Spectrum Disorder (ASD) according to Diagnostic and Statistical Manual of Mental Disorders 4t Edition, (DMS-IV). The severity of the autistics symptomatologiy had been measured by the Childhood Autism Rating Scale (SCARS). We collected urine samples before and after the DMSA challenge test, comparing urine metal output. We also compared the results of the DMSA detoxification(=the urine challenge test) with behavioral effects, typical for ASD.*

Results: *The DMSA challenge test increased the urine metal output for a number of potentially toxic metals. Statistically significant difference were noted between the baseline urine and DMSA challenge test regarding the level of cadmium, mercury, and lead (P=0.006, P=0.049, and P=0.008 respectively). We also noted that behavioral effects, typical for ASD (autism spectrum disorders) were reduced with this method of detoxification. A comparison between CARS Subscales and Total Score before and after a 6-month chelation program showed greatest improvements for Verbal and nonverbal communication (P <0.001), Taste, Smell and Touch (P 0.001) and Relating to People (P 0.005). Other improvements were noted for Adaptation to Change and Improvement.*

Conclusion: *DMSA chelation increased the urinary output of toxic and neurotoxic metals. Our data supports evidence that detoxification treatment with oral DMSA has beneficial effect on ASD patients.*

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Concerns Continue Over Mercury and Autism

To the Editors:

Stehr-Green et al.[1] have misrepresented my work and confused the debate over autism and mercury exposure with ecologic data from Sweden and Denmark. Their report has many flaws. Four stand out.

Their description of the California data promotes complacency regarding autism rates. For an Institute of Medicine (IOM) review,[2] I presented an ecologic analysis of autism rates and mercury exposure demonstrating an association between rising autism rates in California and mercury exposure in childhood vaccines. In their re-use of my charts, the authors claimed incorrectly that the California data represented the larger class of "autism-like disorders." California prevalence rates were reported based solely on autism cases.[3,4] The authors' suggestion minimized the severity of the California situation. These high and rising autism rates point to a public health emergency, and require accurate measurement and precise classification.

Their autism cases account for a fraction of the autism population. The large majority of autism cases are found in outpatient populations. Yet, the analyses in Sweden (exclusively) and Denmark (for two thirds of the study period) rely on inpatient populations. One recent Danish study[5] revealed that 93% of autistic records were for outpatients. Clearly, the small remaining group of inpatient registrations has little value in trend assessment.

Their rate and exposure assessments contain multiple errors. These flaws are too numerous to mention here. (For a more detailed criticism of the Danish and Swedish analyses and a longer version of this letter, go to www.safeminds.org/.) Despite these flaws, they claim, inappropriately, that the choice of Swedish and Danish sources was based on "high quality records."

Their interpretation of the autism–mercury hypothesis is incorrect. Based on these flawed trend assumptions, the authors use the shift in Sweden and Denmark to Thimerosal®-free vaccines in an attempt to falsify the autism–mercury hypothesis. Absent a clear increase in autism rates in Denmark and Sweden, this attempt fails. The autism-mercury hypothesis I tested was that *increases* in mercury exposure are associated with *increases* in autism rates. Reductions in comparatively low Thimerosal® exposures need not produce decreasing autism rates in stable, low-prevalence populations for the autism–mercury hypothesis to hold.

Having performed the ecologic analysis with which the authors started, I fully recognize its failings. I do not wish to stand in defense of ecologic analysis. The authors' attempts at trend analysis demonstrate the dangers of misusing ecologic analysis, especially when relying on shifting data sources and incomplete time series. Resources should flow instead to primary research.

Credible evidence points to rapidly rising U.S. autism rates.[3] Mercury exposure is temporally,[1] epidemiologically,[6] and clinically[7] associable with U.S. autism cases and may help explain these increases. The IOM has called for an active research program[2] that did not include ecologic speculations. Independent scientists should heed their recommendations, remain concerned over the autism-mercury connection and investigate further using proper methods.

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Authors' Reply to Mr. Blaxill's "Concerns Continue Over Mercury and Autism"

In reply:

Our intent in undertaking the investigation to which Mr. Blaxill refers was to further examine the alleged

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Thimerosal and autism? A plausible hypothesis that should not be dismissed☆

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Summary The autism–mercury hypothesis first described by Bernard et al. has generated much interest and controversy. The Institute of Medicine (IOM) reviewed the connection between mercury-containing vaccines and neurodevelopmental disorders, including autism. They concluded that the hypothesis was biologically plausible but that there was insufficient evidence to accept or reject a causal connection and recommended a comprehensive research program. Without citing new experimental evidence, a number of observers have offered opinions on the subject, some of which reject the IOM's conclusions. In a recent review, Nelson and Bauman argue that a link between the preservative thimerosal, the source of the mercury in childhood vaccines, is improbable. In their defense of thimerosal, these authors take a narrow view of the original hypothesis, provide no new evidence, and rely on selective citations and flawed reasoning. We provide evidence here to refute the Nelson and Bauman critique and to defend the autism–mercury hypothesis.

Introduction

In 1999, the US Public Health Service and the American Academy of Pediatrics (AAP) called for the reduction or elimination of the ethylmercury preservative thimerosal from vaccines, saying that the cumulative amount of mercury in infant vaccines exceeded US Environmental Protection Agency (EPA) guidelines for methylmercury [1]. In 2000, Bernard et al. published an extensive literature review which outlined the shared traits and biological abnormalities between mercury poisoning and autism. They suggested that many cases of idiopathic autism may be induced by early mercury exposure and represent an unrecognized mercurial syndrome. They further postulated that genetic and non-genetic factors establish susceptibility whereby mercury's adverse effects do not occur in all children exposed to mercury [2,3]. Since then, the topic has generated a great deal of controversy. In 2001, the IOM reviewed the science literature on thimerosal and found insufficient evidence to accept or reject an association between thimerosal and neurodevelopmental disorders but found the hypothesis ''biologically plausible''. The IOM committee recommended a comprehensive program of research to resolve the

☆ A response to: Nelson KB, Bauman ML. Thimerosal and autism? Pediatrics 2003;111(3):674–79.

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REVIEW ARTICLE

A POSSIBLE CENTRAL MECHANISM IN AUTISM SPECTRUM DISORDERS, PART 1

Russell L. Blaylock, MD

The autism spectrum disorders (ASD) are a group of related neurodevelopmental disorders that have been increasing in incidence since the 1980s. Despite a considerable amount of data being collected from cases, a central mechanism has not been offered. A careful review of ASD cases discloses a number of events that adhere to an immunoexcitotoxic mechanism. This mechanism explains the link between excessive vaccination, use of aluminum and ethylmercury as vaccine adjuvants, food allergies, gut dysbiosis, and abnormal formation of the developing brain.

It has now been shown that chronic microglial activation is present in autistic brains from age 5 years to age 44 years. A considerable amount of evidence, both experimental and clinical, indicates that repeated microglial activation can initiate priming of the microglia and that subsequent stimulation can produce an exaggerated microglial response that can be prolonged.

It is also known that one phenotypic form of microglia activation can result in an outpouring of neurotoxic levels of the excitotoxins, glutamate and quinolinic acid. Studies have shown that careful control of brain glutamate levels is essential to brain pathway development and that excesses can result in arrest of neural migration, as well as dendritic and synaptic loss.

It has also been shown that certain cytokines, such as TNF-α, can, via its receptor, interact with glutamate receptors to enhance the neurotoxic reaction. To describe this interaction I have coined the term *immunoexcitotoxicity,* which is described in this article. (*Altern Ther Health Med.* 2008;14(6):46-53.)

Russell L. Blaylock, MD, is a retired neurosurgeon and visiting professor of biology at Belhaven College, Jackson, Mississippi.

Editor's note: The following is part 1 of a 3-part series. Part 2 will appear in the Jan/Feb 2009 issue of Alternative Therapies in Health and Medicine.

Autism spectrum disorders (ASD) are an increasingly common group of neurodevelopmental disorders without a clearly defined cause. This spectrum of disorders is characterized by a collection of neurobehavioral and neurological dysfunctions often occurring before age 36 months, which include a loss of eye contact, deficiencies in socialization, abnormal theory of mind function, language dysfunction, repetitive behaviors, and some difficulties with executive prefrontal lobe functions.[1,2]

The disorder has a prevalence of males to females of 4:1. A regressive loss of developmental skills occurs in 30%, most often between the ages of 18 months and 24 months. It also has been noted that autistic boys are more likely to experience an early onset of puberty.[3-5] Recent epidemiological evidence indicates a rapid rise in the prevalence of autism, with a 1 in 150 to a 1 in 160 incidence.

Neuropathological studies have shown abnormalities in the architecture of the autistic brain affecting cortical, subcortical, limbic, and cerebellar structures.[6-8] One of the most consistent findings has been hypoplasia of the inferior vermis of the cerebellum with variable but substantial loss of Purkinje cells in the cerebellar cortex.

The bulk of the evidence indicates that immune factors play a major role in these disorders.[9-11] Likewise, abundant evidence implicates mercury neurotoxicity from previously high levels of ethylmercury used as a preservative (thimerosal) in a number of childhood vaccines, as well as other sources of mercury.[12,13]

A host of other observations related to ASD has been aired, including abnormalities in organic acids, opioid-like substances from gliadin and gluten metabolism, intestinal dysbiosis, and trace element imbalances. A strong genetic influence is also known to exist.[14]

Neuroscience has discovered one mechanism that explains most of the findings in ASD: the excitotoxic cascade. New studies have linked a number of seemingly unrelated events to this cascade, such as immune activity, neurohormone abnormalities, and a host of biochemical events.[15,16] Examination of the pieces to this puzzle demonstrate that most fit well into this mechanism.

THE EXCITOTOXIC CASCADE

In 1957, Lucas and Newhouse discovered that monosodium glutamate (MSG)–exposed rats developed degeneration of the inner ganglion layers of the retina.[17] John Olney in 1969 discovered that the food additive MSG could produce delayed neuron death when animals were fed the substance in higher concentrations.[18] He observed not only destruction of the animals' retinal neurons but also destruction of selected nuclei in the hypothalamus and other brain structures. He coined the name *excitotoxin* based on the early observation that the neurons seemed to excite themselves to death in a delayed manner.

The glutamate receptor system consists of 3 ionotropic receptors

REVIEW ARTICLE

A POSSIBLE CENTRAL MECHANISM IN AUTISM SPECTRUM DISORDERS, PART 2: IMMUNOEXCITOTOXICITY

Russell L. Blaylock, MD

In this section, I explore the effects of mercury and inflammation on transsulfuration reactions, which can lead to elevations in androgens, and how this might relate to the male preponderance of autism spectrum disorders (ASD). It is known that mercury interferes with these biochemical reactions and that chronically elevated androgen levels also enhance the neurodevelopmental effects of excitotoxins. Both androgens and glutamate alter neuronal and glial calcium oscillations, which are known to regulate cell migration, maturation, and final brain cytoarchitectural structure. Studies have also shown high levels of DHEA and low levels of DHEA-S in ASD, which can result from both mercury toxicity and chronic inflammation.

Chronic microglial activation appears to be a hallmark of ASD. Peripheral immune stimulation, mercury, and elevated levels of androgens can all stimulate microglial activation. Linked to both transsulfuration problems and chronic mercury toxicity are elevations in homocysteine levels in ASD patients. Homocysteine and especially its metabolic products are powerful excitotoxins.

Intimately linked to elevations in DHEA, excitotoxicity and mercury toxicity are abnormalities in mitochondrial function. A number of studies have shown that reduced energy production by mitochondria greatly enhances excitotoxicity. Finally, I discuss the effects of chronic inflammation and elevated mercury levels on glutathione and metallothionein. (*Altern Ther Health Med.* 2009;15(1):60-67.)

Russell L. Blaylock, MD, is a retired neurosurgeon and professor of biology at Belhaven College, Jackson, Mississippi.

Editor's note: The following is part 2 of a 3-part series. Part 3 will appear in the Mar/Apr 2009 issue of Alternative Therapies in Health and Medicine.

EXCESSIVE ANDROGENS AND AUTISM

There is strong evidence that mercury exposure in humans increases androgen levels. For example, Barregård et al reported that there was a significant correlation between increasing concentration of mercury in chloralkali workers and testosterone levels.[1] Animal studies also show a link between sex steroid production and mercury dosing.[2] Studies have also shown a link between elevated prenatal testosterone,[3] postnatal serum testosterone,[4] and autism spectrum disorders.

As to the mechanism of testosterone elevation by mercury exposure, it has been suggested that Hg^{2+} directly causes a defect in adrenal steroid biosynthesis by inhibiting the activity of 21 alpha-hydroxylase,[5] while others have suggested inactivation of hydroxysteroid steroid sulfotransferase either directly[6] or by way of inflammation.[7] It has also been shown that DHEA-S, the proposed storage form of active DHEA, is also significantly lowered in autistic disorders.[8]

Kim et al have shown that even very small doses of LPS (1 nmoL) can dramatically decrease the levels of mRNA for SULT2A1 and PAPSS2, which are responsible for sulfonation of a number of endogenous hydroxysteroids, bile acid, and xenobiotics as well as sulfonation of DHEA to DHEA-S.[7] Normally, DHEA-S plasma levels are 300- to 500-fold higher than DHEA levels. Kim et al found that TNF-α and IL-1ß were responsible for the decrease. Unlike in autistic patients, DHEA levels were not increased in LPS-exposed animals, which can occur with mercury toxicity. Reductions in DHEA-S are common with other chronic inflammatory disorders, such as rheumatoid arthritis.[9]

In keeping with the finding of a defect in transsulfuration, one frequently sees associated elevations in androgens and elevations in homocysteine. For instance, several workers have found elevated levels of homocysteine in cases of polycystic ovary syndrome.[10,11] Normally, men have higher homocysteine levels than women, thought to be secondary to higher androgen levels.[12]

Androgen excess interferes with the conversion of homocysteine to cysthathionine, which by conversion to cysteine becomes a major source of glutathione.[13] Thus androgen excess can not only raise homocysteine levels, it can lower glutathione, a major antioxidant in brain. Other pathways in the methionine cycle are also affected, which may partially explain the significant reduction in methionine seen in autistic children, as well as s-adenosyl methionine levels.[4,14]

James et al found not only low total glutathione levels in autistic subjects but also oxidized glutathione levels that were 2-fold higher, which strongly indicate oxidative stress.[14] Several of

Immune-Glutamatergic Dysfunction as a Central Mechanism of the Autism Spectrum Disorders

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Abstract: Despite the great number of observations being made concerning cellular and the molecular dysfunctions associated with autism spectrum disorders (ASD), the basic central mechanism of these disorders has not been proposed in the major scientific literature. Our review brings evidence that most heterogeneous symptoms of ASD have a common set of events closely connected with dysregulation of glutamatergic neurotransmission in the brain with enhancement of excitatory receptor function by pro-inflammatory immune cytokines as the underlying mechanism. We suggest that environmental and dietary excitotoxins, mercury, fluoride, and aluminum can exacerbate the pathological and clinical problems by worsening excitotoxicity and by microglial priming. In addition, each has effects on cell signaling that can affect neurodevelopment and neuronal function. Our hypothesis opens the door to a number of new treatment modes, including the nutritional factors that naturally reduce excitotoxicity and brain inflammation.

Keywords: Autism spectrum disorders, excitotoxicity, fluoride, glutamatergic neurotransmission, inflammation, mercury, microglia, cytokines.

1. INTRODUCTION

We have witnessed an alarming increase in the incidence of ASD with rates increasing from 1 in 2,325 births prior to the 1980s to 1 in 101 births today. For male children, the incidence is now 1 in 67 births in some areas of the USA [1]. ASD are a group of related neurodevelopmental disorders, which includes autism, pervasive developmental disorder-not-otherwise specified (PDD-NOS), Asperger syndrome, Rett´s syndrome, and childhood disintegrative syndrome. The terms ASD and autism are used interchangably. ASD are characterized by a collection of neurobehavioral and neurological dysfunctions, often occurring before age 36 months [2-4]. Despite the great array of observations being made at the cellular and the molecular levels, no one has proposed an integrative and unifying mechanism to explain the heterogenous symptoms and etiology of ASD. Given the major role of glutamate in brain development, some authors have hypothesized that alterations of glutamatergic neurotransmission play a role in the pathophysiology of autism [5-10]. The hyperglutamatergic hypothesis of autism has been discussed recently [11, 12] focusing on findings of increased serum level of glutamate in children and adults with ASD [13, 9], the reduction of the levels of rate-limiting enzymes glutamate acid decarboxylase 65 and 67 (GAD65 and GAD67) and the increased gliosis in the brains of autistic subjects [14, 15].

A number of studies leave little question that there is a genetic propensity for autism risk. Studies showing higher incidence rates of 60 to 90% in monozygotic twins versus 0 to 6% in dizygotic twins suggest a herediability of over 90% [2]. Data from whole genome screening of multiplex families (having more than one autistic child) strongly suggest that 10 or more genes interact to cause classic autism [16]. Recent research of the autism genome supports further the view that abnormalities in genes connected with glutamate receptors (GluR) and regulation of glutamate pathways may be directly involved in ASD pathology [17]. A significant association between GluR6 gene, located on chromosome 6q21, and autism were found [5, 18, 19]. GluR6 genes control a member of the ionotropic receptor kainate family, which plays a major role in brain development. Strong evidence points to a mutation in chromosome loci 7q31 in both autism and language disorders [20]. The sequence on chromosome 11p12-13 has been linked to glutamate transport proteins. The neurexin-1 gene (*NRXN1)* has been shown to play a fundamental role in synaptogenesis and synaptic maintenance, as well as Ca^{2+} channel and NMDA receptor recruitment [21].

Serajee *et al.* [22] demonstrated from a study of 196 families having autistic children, a high incidence of mutation of the GRM8 gene controlling the metabotropic GluR8 receptor subunit, which negatively modulates glutamate neurotransmission. Mutation of this gene increases glutamate hyperactivity and thus excitotoxicity. This receptor subunit is located on a number of anatomical areas of the brain affected in autism, including the lateral reticular thalamic nucleus, pyriform cortex and to a lesser degree the cerebellum, caudate and hippocampus [23]. Ramanathan *et al.* [24] detected abnormalities in genes controlling AMPA receptors (GluR2) as well as glycine receptors (GLRA3 and GLRB), which play a critical role in inotropic GluR control, in a single case of autism. It is obvious from these studies that genetic influence on glutamate function is playing a role in the ASD.

In this paper we offer the explanation of potential etiology of ASD as dysregulation of glutamatergic neurotransmission, with underlying interactions between chronic microglial activation, and the excitotoxic cascade playing the central role. Table **1** gives observed alterations in ASD, which may be connected with dysfunctions of glutamatergic neurotransmission. Moreover, we suggest that the increasing prevalence of ASD during the last decades might reflect the

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A Case-Control Study of Mercury Burden in Children with Autistic Spectrum Disorders

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ABSTRACT

Large autism epidemics have recently been reported in the United States and the United Kingdom. Emerging epidemiologic evidence and biologic plausibility suggest an association between autistic spectrum disorders and mercury exposure.

This study compares mercury excretion after a three-day treatment with an oral chelating agent, meso-2,3-dimercaptosuccinic acid (DMSA), in children with autistic spectrum disorders and a matched control population. Overall, urinary mercury concentrations were significantly higher in 221 children with autistic spectrum disorders than in 18 normal controls (Relative Increase (RI) = 3.15; $P < 0.0002$). Additionally, vaccinated cases showed a significantly higher urinary mercury concentration than did vaccinated controls (RI = 5.94; $P < 0.005$). Similar urinary mercury concentrations were observed among matched vaccinated and unvaccinated controls, and no association was found between urinary cadmium or lead concentrations and autistic spectrum disorders.

The observed urinary concentrations of mercury could plausibly have resulted from thimerosal in childhood vaccines, although other environmental sources and thimerosal in Rh_o(D) immune globulin administered to mothers may be contributory.

Regardless of the mechanism by which children with autistic spectrum disorders have high urinary mercury concentrations, the DMSA treatment described in this study might be useful to diagnose their present burden of mercury.

KEY WORDS: autism, autistic spectrum disorders, chelation, DMSA, mercury, thimerosal

Background

Recent studies have analyzed the prevalence of autism from the mid-1980s through 2002 in the United States and the United Kingdom.[1-5] The prevalence of autism is estimated to have risen from one in about 2,500 children in the mid-1980s to as common as one in 150 by 2002. Further, since all of these studies find the prevalence of autism in males to be four times that of females, the male prevalence of this disorder exceeds one in 100. These studies show that the rise in the prevalence in autism is genuine and not the result of population migration, differences in diagnostic criteria, or other potential confounders.

In 2001, the Institute of Medicine (IOM) of the United States National Academy of Sciences[6] determined that a link between mercury from thimerosal contained in childhood vaccines and the recent dramatic increase in neurodevelopment disorders is biologically plausible. Recent studies demonstrate a strong epidemiologic link between exposure to mercury from thimerosal contained in childhood vaccines and neurodevelopment disorders.[7-9]

The purpose of this study was to evaluate the concentration of mercury in the urine following a three-day treatment with an oral chelating agent, meso-2,3-dimercaptosuccinic acid (DMSA), in children with autistic spectrum disorders in comparison to a control population. Forman et al.[10] have reported on the use of oral treatment with DMSA in children exposed to metallic mercury. The authors found that oral chelation with DMSA produced a significant mercury diuresis in these children. They observed no adverse side effects of treatment. The authors concluded that DMSA appears to be an effective and safe chelating agent for treatment of pediatric overexposure to metallic mercury. In addition, extensive literature supports its safety in the chelation of lead from exposed children.

Methods

This study is a retrospective analysis of 221 consecutive children with previously established autism spectrum disorders referred and admitted to the International Child Development Resource Center (ICDRC). Each child had been diagnosed with autism (DSM-IV 299.00) or pervasive developmental disorder (DSM-IV 299.80) by outside physicians. A control population of 18 children was also identified without autism spectrum disorders in themselves or among their siblings or their first-degree family members. These healthy children presented to the ICDRC for elective determination of their levels of environmental mercury exposure at the request of their families, and are included here for case comparison. The Arizona State University Institutional Review Board approved our retrospective examination of cases and controls in this study.

All children were examined to exclude those who had dental amalgams. Among the 221 cases, all had received their full scheduled childhood immunizations appropriate for their respective ages. Among the 18 controls, 10 children had received their full childhood immunization schedules, and 8 children had received no childhood immunizations because of religious objections.

Informed consent was obtained from both cases and controls for DMSA chelation treatment. Controls and cases were both challenged with a three-day oral treatment of DMSA (10 mg/kg per dose given three times daily). After the ninth dose, the first voided morning urine was collected (when possible), or an overnight urine collection bag was worn. All laboratory analyses were performed by the Doctors' Data, Inc., in Chicago, Ill. The response to DMSA was measured as micrograms of mercury per gram of creatinine using inductively coupled mass spectrometry, and creatinine was measured using the Jaffe method. The laboratory was not informed whether the specimens were from cases or controls.

In addition to the overall excretion data, several epidemiologic case-control studies were conducted using the available populations. First, it was possible to match 88 cases against 16 controls for age (within one year) and sex, and overall post-DMSA urinary

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SHORT COMMUNICATION

Gender-selective toxicity of thimerosal ☆

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Abstract

A recent report shows a correlation of the historical use of thimerosal in therapeutic immunizations with the subsequent development of autism; however, this association remains controversial. Autism occurs approximately four times more frequently in males compared to females; thus, studies of thimerosal toxicity should take into consideration gender-selective effects. The present study was originally undertaken to determine the maximum tolerated dose (MTD) of thimersosal in male and female CD1 mice. However, during the limited MTD studies, it became apparent that thimerosal has a differential MTD that depends on whether the mouse is male or female. At doses of 38.4–76.8 mg/kg using 10% DMSO as diluent, seven of seven male mice compared to zero of seven female mice tested succumbed to thimerosal. Although the thimerosal levels used were very high, as we were originally only trying to determine MTD, it was completely unexpected to observe a difference of the MTD between male and female mice. Thus, our studies, although not directly addressing the controversy surrounding thimerosal and autism, and still preliminary due to small numbers of mice examined, provide, nevertheless, the first report of gender-selective toxicity of thimerosal and indicate that any future studies of thimerosal toxicity should take into consideration gender-specific differences.

Keywords: Thimerosal; Thimerosal toxicity; Gender-selective toxicity; Maximum tolerated dose; Autism

Introduction

Thimerosal is an organic compound that contains mercury and has been used historically as a preservative in vaccines and pharmaceutical products. The breakdown product, ethylmercury, in thimerosal-preserved childhood vaccines has been suggested to be neurotoxic and to contribute to the etiology of neurodevelopmental disorders, including autism; however, this supposition is highly controversial (Mutter et al., 2005; Geier et al., 2007; Ng et al., 2007; Zareba et al., 2007; Thompson et al., 2007, Schechter and Grether, 2008). It has, however, been shown that mercury and thimerosal administration results in the decreased production of

☆ *Ethical Statement*: All animal studies were performed under an approved animal use protocol (AUP) for the care and use of animals (mice) by Nucro-Technics, 2000 Ellesmere Road, Scarborough, Ontario, Canada. Nucro-Technics is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and the Canadian Council on Animal Care. The study was conducted under the direction of Dr. Albert Licollari, DVM, Ph.D.

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Comparison of Blood and Brain Mercury Levels in Infant Monkeys Exposed to Methylmercury or Vaccines Containing Thimerosal

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Thimerosal is a preservative that has been used in manufacturing vaccines since the 1930s. Reports have indicated that infants can receive ethylmercury (in the form of thimerosal) at or above the U.S. Environmental Protection Agency guidelines for methylmercury exposure, depending on the exact vaccinations, schedule, and size of the infant. In this study we compared the systemic disposition and brain distribution of total and inorganic mercury in infant monkeys after thimerosal exposure with those exposed to MeHg. Monkeys were exposed to MeHg (via oral gavage) or vaccines containing thimerosal (via intramuscular injection) at birth and 1, 2, and 3 weeks of age. Total blood Hg levels were determined 2, 4, and 7 days after each exposure. Total and inorganic brain Hg levels were assessed 2, 4, 7, or 28 days after the last exposure. The initial and terminal half-life of Hg in blood after thimerosal exposure was 2.1 and 8.6 days, respectively, which are significantly shorter than the elimination half-life of Hg after MeHg exposure at 21.5 days. Brain concentrations of total Hg were significantly lower by approximately 3-fold for the thimerosal-exposed monkeys when compared with the MeHg infants, whereas the average brain-to-blood concentration ratio was slightly higher for the thimerosal-exposed monkeys (3.5 ± 0.5 vs. 2.5 ± 0.3). A higher percentage of the total Hg in the brain was in the form of inorganic Hg for the thimerosal-exposed monkeys (34% vs. 7%). The results indicate that MeHg is not a suitable reference for risk assessment from exposure to thimerosal-derived Hg. Knowledge of the toxicokinetics and developmental toxicity of thimerosal is needed to afford a meaningful assessment of the developmental effects of thimerosal-containing vaccines. *Key words:* brain and blood distribution, elimination half-life, ethylmercury, infant nonhuman primates, methylmercury, thimerosal. *Environ Health Perspect* 113:1015–1021 (2005). doi:10.1289/ehp.7712 available via *http://dx.doi.org/* [Online 21 April 2005]

Public perception of the safety and efficacy of childhood vaccines has a direct impact on immunization rates (Biroscak et al. 2003; Thomas et al. 2004). The current debate linking the use of thimerosal in vaccines to autism and other developmental disorders [Institute of Medicine (IOM) 2001, 2004] has led many families to question whether the potential risks associated with early childhood immunizations may outweigh the benefits (Blaxill et al. 2004; SafeMinds 2005). Thimerosal is an effective preservative that has been used in the manufacturing of vaccines since the 1930s. Thimerosal consists of 49.6% mercury by weight and breaks down in the body to ethylmercury and thiosalicylate (Tan and Parkin 2000). Recent reports have indicated that some infants can receive ethylmercury (in the form of thimerosal) at or above the U.S. Environmental Protection Agency (EPA) guidelines for methylmercury exposure (U.S. EPA 2005), depending on the exact vaccinations, schedule, and size of the infant (Ball et al. 2001). Clements et al. (2000) calculated that children receive 187.5 μg of ethylmercury from thimerosal-containing vaccines given over the first 14 weeks of life. According to the authors, this amount approaches or, in some cases, exceeds the U.S. EPA guidelines for MeHg exposure during pregnancy (0.1 μg/kg/day). Other estimates (Halsey 1999) have indicated that the schedule could provide repeated doses of ethylmercury from approximately 5 to 20 μg/kg over the first 6 months of life. Studies in preterm infants indicate that blood levels of Hg after just one vaccination (hepatitis B) increase by > 10-fold to levels above the U.S. EPA guidelines (Stajich et al. 2000).

The U.S. EPA guidelines for MeHg (U.S. EPA 2005) are based on several decades of studies of humans and animal models of developmental toxicity (Burbacher et al. 1990a; National Research Council 2000). Because few data exist for ethylmercury, the use of the MeHg guidelines would seem appropriate if the two compounds have similar toxicokinetic profiles and neurodevelopmental effects. The results from the few studies that have provided a direct comparison of these two compounds have been reviewed recently by Magos (2003), who concluded that *a*) Hg clears from the body faster after the administration of ethylmercury than after the administration of MeHg; *b*) the brain-to-blood Hg concentration ratio established for MeHg will overestimate Hg in the brain after exposure to ethylmercury; and *c*) because ethylmercury decomposes faster than MeHg, the risk of brain damage is less for ethylmercury than for MeHg. These conclusions are based on only a few studies, none of which included measurements of both blood and brain Hg levels in infant subjects.

We initiated the present study in order to directly compare the blood and brain levels of Hg in infant nonhuman primates exposed orally to MeHg or via intramuscular (im) injections of vaccines containing thimerosal. Nonhuman primates have been used extensively in previous studies of MeHg toxicokinetics and developmental neurotoxicity (Burbacher et al. 1986, 1990b; Gunderson et al. 1986, 1988; Rice and Gilbert 1982, 1990, 1995; Stinson et al. 1989; Vahter et al. 1994, 1995). The routes of administration (oral for MeHg and im injection for thimerosal-containing vaccines) were chosen to mimic the two routes of Hg exposure for humans. The dosages and schedule of administration of Hg were chosen to be comparable with the current immunization schedule for human newborns, taking into consideration the faster growth (~ 4 to 1) of the macaque infant (Gunderson and Sackett 1984). The results of the present study provide important new information regarding the comparative toxicokinetics of these two compounds in newborns and infants.

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A systematic study of the disposition and metabolism of mercury species in mice after exposure to low levels of thimerosal (ethylmercury)☆

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ABSTRACT

Thimerosal (TM) is an ethylmercury (etHg)-containing preservative used in some vaccines despite very limited knowledge on the kinetics and direct interaction/effects in mammals' tissues after exposure. Thus, this study aimed to evaluate the kinetics of Hg species in mice in a time course analysis after intramuscular injection of TM, by estimating Hg half-lives in blood and tissues. Mice were exposed to one single intramuscular dose of 20 μg of Hg as TM. Blood, brain, heart, kidney and liver were collected at 0.5 hour (h), 1 h, 8 h, 16 h, 144 h, 720 h and 1980 h after TM exposure ($n=4$). Hg species in animal tissues were identified and quantified by speciation analysis *via* liquid chromatography hyphenated with inductively coupled mass spectrometry (LC–ICP-MS). It was found that the transport of etHg from muscle to tissues and its conversion to inorganic Hg (inoHg) occur rapidly. Moreover, the conversion extent is modulated in part by the partitioning between EtHg in plasma and in whole blood, since etHg is rapidly converted in red cells but not in a plasma compartment. Furthermore, the dealkylation mechanism in red cells appears to be mediated by the Fenton reaction (hydroxyl radical formation). Interestingly, after 0.5 h of TM exposure, the highest levels of both etHg and inoHg were found in kidneys (accounting for more than 70% of the total Hg in the animal body), whereas the brain contributed least to the Hg body burden (accounts for $<1.0\%$ of total body Hg). Thirty days after TM exposure, most Hg had been excreted while the liver presented the majority of the remaining Hg. Estimated half-lives (in days) were 8.8 for blood, 10.7 for brain, 7.8 for heart, 7.7 for liver and 45.2 for kidney. Taken together, our findings demonstrated that TM (etHg) kinetics more closely approximates Hg^{2+} than methylmercury (meHg) while the kidney must be considered a potential target for etHg toxicity.

1. Introduction

Thimerosal (TM), which contains ethylmercury (etHg), has been widely used as a preservative in a number of drug products, including vaccines, to help prevent life-threatening contamination with microbes (Tan and Parkin, 2000). However, the potential neurotoxic effects of organomercurial compounds, even at low exposures (Lebel et al., 1998; Berman et al., 2008; Delong, 2011; Bose et al., 2012; Petroni et al., 2012; Ida-Eto et al., 2013), have provoked concerns about the use of thimerosal in vaccines and other products (Clements et al., 2000; Ball et al., 2001).

The toxic properties of Hg compounds are directly related to the chemical form of the element. In general, exposure to organic forms of Hg is associated with nervous system damage, while inorganic forms are closely connected to renal damage (Clarkson and Magos, 2006). However, the toxicokinetics and potential toxic properties of TM (etHg) are mostly unknown (WHO, 2012).

Due to the lack of information about the behavior of TM in the mammalian body, the initial risk assessments for etHg were based on studies of oral methylmercury (meHg) toxicity. However, recent

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Thimerosal Induces Apoptotic and Fibrotic Changes to Kidney Epithelial Cells *In Vitro*

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ABSTRACT: Thimerosal is an ethyl mercury-containing compound used mainly in vaccines as a bactericide. Although the kidney is a key target for mercury toxicity, thimerosal nephrotoxicity has not received the same attention as other mercury species. The aim of this study was to determine the potential cytotoxic mechanisms of thimerosal on human kidney cells. Human kidney proximal tubular epithelial (HK2) cells were exposed for 24 h to thimerosal (0–2 μM), and assessed for cell viability, apoptosis, and cell proliferation; expression of proteins Bax, nuclear factor-κB subunits, and transforming growth factor-β1 (TGFβ1); mitochondrial health (JC-1, MitoTracker Red CMXRos); and fibronectin levels (enzyme-linked immunosorbent assay). Thimerosal diminished HK2 cell viability and mitosis, promoted apoptosis, impaired the mitochondrial permeability transition, enhanced Bax and TGFβ1 expression, and augmented fibronectin secretion. This is the first report about kidney cell death and pro-fibrotic mechanisms promoted by thimerosal. Collectively, these *in vitro* results demonstrate that (1) thimerosal induces kidney epithelial cell apoptosis via upregulating Bax and the mitochondrial apoptotic pathway, and (2) thimerosal is a potential pro-fibrotic agent in human kidney cells. We suggest that new evidence on toxicity as well as continuous surveillance in terms of fibrogenesis is required concerning thimerosal use. Environ Toxicol 30: 1423–1433, 2015.

Keywords: kidney; thimerosal; ethyl mercury; apoptosis; mitochondrial dysfunction; fibrosis; toxicity

INTRODUCTION

Thimerosal is a mercury (Hg)-containing compound used mainly in vaccines—particularly in developing nations—as a bactericide composed of ∼50% ethyl mercury (etHg) (w/w) (Tan and Parkin, 2000; Durrheim and Poland, 2013). Exposure to thimerosal has been associated with the development of neurological disorders but the debate on this causality is still ongoing (Nelson and Bauman, 2003; Gallagher and Goodman, 2010; Hewitson et al., 2010; Delong, 2011; Garcia-Fernandez et al., 2013). Its contribution to diseases in other organs is also still under investigation.

Currently, most of the studies have focused on etHg/thimerosal effects upon neurons and other cell types from the nervous tissue (Baskin et al., 2003; Humphrey et al., 2005; Sharpe et al., 2012). Nonetheless, the kidney is a key target organ for Hg toxicity (Jan et al., 2011; Al-Saleh et al., 2012). Most reported evidence on kidney toxicity comes from studies of methyl Hg (meHg) and inorganic Hg. These investigations have indicated an outcome of increased apoptosis. Mechanisms have included changes in the redox state

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REVIEW ARTICLE

THE HISTORY OF VACCINATIONS IN THE LIGHT OF THE AUTISM EPIDEMIC

Stephanie F. Cave, MD, MS, FAAFP

Autism has been characterized as a behavioral disorder since it was first described by Leo Kanner in 1943. The number of autistic children has increased over the last decade. The incidence of autism was 1 in 10 000 before the 1970s and has steadily increased to 1 in 150 in 2008 with a male:female predominance of 4:1. The cause of this epidemic has remained unknown, but several hypotheses have been studied. Many of these suggest an environmental trigger, such as the ethyl mercury contained in the preservative thimerosal, which has been used in vaccines since 1931. Other possible triggers associated with vaccinations are chemical toxins and live viruses. James has published studies suggesting a genetic predisposition in the families of autistic children, exposing them to a deficiency in glutathione and an inability to detoxify heavy metals. Vargas has shown autism to encompass ongoing inflammation in the brains of autistic children. The Hannah Poling vaccine decision was a landmark case. Poling's family was awarded funds for ongoing medical care of an autistic child who was found to have mitochondrial dysfunction exacerbated by vaccines that left her with autistic behavior and seizures. Several studies have emerged supporting the fact that a significant number of autistic children do have mitochondrial dysfunction. The impact that the Poling case will have on the ability of parents of autistic children to gain access to funds to enable them to properly care for their children remains to be seen. (*Altern Ther Health Med.* 2008;14(6):54-57.)

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English scientist Edward Jenner used a scientific approach to vaccination in the 18th century by inoculating people with cowpox to protect them against smallpox. This strategy helped but was short-lived because of the possibility of contamination. Louis Pasteur developed the first rabies vaccine for humans in 1885 and introduced the concept of attenuation or weakening the virus in the vaccine to avoid injuring the recipient.

The polio vaccines by Sabin and Salk followed. The injectable vaccine was used in 1955, sparking the use of mass vaccination in a free program for the public through the Poliomyelitis Vaccination Assistance Act. From 1906 to 1946, the diphtheria, tetanus, and pertussis vaccines were developed. The DTP combination vaccine was made available to the public in 1946.[1]

The measles vaccine became available in 1963, followed in 1968 by the mumps vaccine and in 1969 by the rubella, or German measles, vaccine. Pneumococcal vaccine became available in 1978, and in 1979 the measles, mumps, and rubella vaccines were marketed as the combination MMR. The 1980s brought into use the hepatitis B and *Haemophilus influenzae* vaccines.

In 1991, the recombinant hepatitis B vaccine was recommended for use in newborns within 24 hours of birth. Also in 1991, the hepatitis B, *Haemophilus influenzae* B, and DTP vaccines were given together to children during the same office visit. All 3 of these contained the preservative thimerosal, which contained ethyl mercury. Additionally, administration of the Rho (D) immune globulin given to Rho (D) negative mothers was moved from postbirth to the 28th week of gestation. The immune globulin contained thimerosal until 2002. From 1991 to 1999, children inadvertently received up to 125 times the safe level of mercury recommended by the Environmental Protection Agency (EPA); this number is determined by the oral methylmercury standard on any given vaccine day that multiple vaccines were given. This level exceeded not only the EPA standard but also the safety standards of the US Food and Drug Administration, the Agency for Toxic Substances and Disease Registry, and the World Health Organization.

HISTORY OF AUTISM

Autism was first described by psychiatrist Leo Kanner, MD, in 1943 as a behavioral disorder of children.[4] Around the same time, Hans Asperger was writing about children who had similar symptoms but no compromised speech. Many professionals and parents of autistic children have watched the number of autistic children rise to epidemic proportions while the toxic levels of ethyl mercury and other toxins persist in vaccines.

Autism has been classified by the disciplines of medicine as a psychiatric illness.[2] Recently, autistic children have displayed symptoms of disease in many of the bodily systems. These include but are not limited to the gastrointestinal, neurological, and immune systems. In many cases there has been a regression in development closely following a round of vaccinations. The vaccinations in question contained a combination of thimerosal, aluminum, live viruses,

Effect of thimerosal on the neurodevelopment of premature rats

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Original article

Background: **This study was undertaken to determine the effect of thimerosal on the neurodevelopment of premature rats.**

Methods: **Thimerosal was injected into premature SD rats at a dose of 32.8, 65.6, 98.4 or 131.2 μg/kg on postnatal day 1. Expression of dopamine D4 receptor (DRD4) and serotonin 2A receptor (5-HT2AR), apoptosis in the prefrontal cortex on post-injection day 49, and learning and memory function were studied and compared with those in a control group injected with saline.**

Results: **Expression of DRD4 and 5-HT2AR and learning function decreased, and apoptosis increased significantly in the 131.2 μg/kg group (P<0.001). Memory function was significantly impaired by 65.6 (P<0.05), 98.4 and 131.2 μg/kg (P<0.001).**

Conclusions: **The negative adverse consequences on neurodevelopment observed in the present study are consistent with previous studies; this study raised serious concerns about adverse neurodevelopmental disorder such as autism in humans following the ongoing worldwide routine administration of thimerosal-containing vaccines to infants.**

World J Pediatr 2013;9(4):356-360

Key words: **dopamine D4 receptor; neurodevelopment; serotonin 2A receptor; thimerosal**

Introduction

Neurological alterations that may result from thimerosal exposure have recently become a hot topic. Thimerosal exposure via vaccination is thought to cause brain disorder.[1] Since there is no appropriate agent to replace, thimerosal is used as a preservative in vaccines. Therefore, it is necessary to determine the appropriate levels of thimerosal for neurodevelopment. Studies[2,3] have been focused on neurological alterations after exposure to thimerosal in rats, but further study is required to demonstrate the acceptable levels of exposure for neurodevelopment.

Rat model is considered feasible for research in intoxication following metal exposure. Learning and memory are important brain functions. And the prefrontal cortex is a critical region receiving stimulation for the development of learning and memory function,[4] which is mainly executed by neurotransmitters. The variants of dopamine D4 receptor (DRD4) are reported to be associated with memory function of rats,[5] whereas serotonin 2A receptor (5-HT2AR) is correlated with impaired episodic memory performance.[6] It was reported that in the human neuroblastoma cell line, thimerosal induced mitochondria-mediated apoptosis.[7]

In the present study, we investigated whether thimerosal could induce alterations in expression of DRD4 and 5-HT2AR, apoptosis of the prefrontal cortex, and learning and memory functions in the premature rats.

Methods

The protocol of this study was approved by the Institutional Ethics Committee of Xi'an Jiaotong University Health Science Center, Xi'an, China. Thirty premature Sprague-Dawley rats (Laboratory Animal Center of Xi'an Jiaotong University Health Science Center) were delivered on day 20 of gestation (term=day 22) by hysterotomy, and they were randomly divided into five groups, with six rats in each group. Thimerosal (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline and injected into the gluteus maximus of four

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Ethylmercury-Induced Oxidative and Endoplasmic Reticulum Stress-Mediated Autophagic Cell Death: Involvement of Autophagosome–Lysosome Fusion Arrest

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ABSTRACT

Ethylmercury (EtHg) is derived from the degradation of thimerosal, the most widely used organomercury compound. In this study, EtHg-induced toxicity and autophagy in the mouse kidney was observed and then the mechanism of toxicity was explored *in vitro* in HK-2 cells. Low doses of EtHg induced autophagy without causing any histopathological changes in mouse kidneys. However, mice treated with high doses of EtHg exhibited severe focal tubular cell necrosis of the proximal tubules with autophagy. EtHg dose-dependently increased the production of reactive oxygen species, reduced the mitochondrial membrane potential, activated the unfolded protein response, and increased cytosolic Ca^{2+} levels in HK-2 cells. Cell death induced by EtHg exposure was caused by autophagy and necrosis. N-acetyl cysteine and 4-phenylbutyric acid attenuated EtHg-induced stress and ameliorated the autophagic response in HK-2 cells. Furthermore, EtHg blocked autophagosome fusion with lysosomes, which was demonstrated via treatment with wortmannin and chloroquine. Low doses of EtHg and rapamycin, which resulted in minimal cytotoxicity, increased the levels of the autophagic SNARE complex STX17 (syntaxin 17)-VAMP8-SNAP29 without altering mRNA levels, but high dose of EtHg was cytotoxic. Inhibition of autophagic flux by chloroquin increased autophagosome formation and necrotic cell death in HK-2 cells. Collectively, our results show that EtHg induces autophagy via oxidative and ER stress and blockade of autophagic flux. Autophagy might play a dual role in EtHg-induced renal toxicity, being both protective following treatment with low doses of EtHg and detrimental following treatment with high doses.

Key words: ethylmercury; ER stress; mitochondrial dysfunction; autophagy; blocking autophagic flux

Mercury is a serious environmental pollutant worldwide; it is produced by industrial processes. It is unique among the heavy metals found in the environment because it takes several physical and chemical forms: elemental mercury, inorganic mercury, and organic mercury (Zalups, 2000). Although the distribution, toxicity, and metabolism of mercury are highly dependent on its chemical form, the primary toxic targets of inorganic and organic mercury compounds are the kidneys and central nervous system, respectively, in humans and animals (Clarkson and Magos, 2006). Methylmercury (MeHg) and ethylmercury (EtHg) are short-chain alkyl mercurial compounds with similar chemical properties. MeHg is known to be one of the most toxic forms of Hg and the most common form of mercury exposure in fish-eating populations. EtHg is derived from the metabolism of

CORRESPONDENCE

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Mercury in infants given vaccines containing thiomersal

Sir—In July 1999, the American Academy of Pediatrics (AAP) published recommendations to minimise exposure to the mercury-based vaccine preservative, thiomersal.[1] A main recommendation was to delay administration of the birth dose of hepatitis B vaccine (containing 12·5 μg mercury) until 6 months of age. This recommendation was made because blood mercury concentrations after injection of thiomersal are inversely related to bodyweight.

Given this relation, I am surprised that Michael Pichichero and colleagues (Nov 30, p 1737)[2] restricted their study to 2-month-old and 6-month-old infants and did not take blood samples within 72 h of vaccination. As shown in figure 1 of their report, the highest blood mercury concentrations were seen in infants 2 months of age and in samples obtained 5–7 days after injection. These results raise the possibility that some infants who received a thiomersal-containing vaccine at birth, as most in the USA did throughout the 1990s, would have had blood mercury concentrations within the first 3 days after vaccination that exceeded the safety threshold value of 29 nmol/L cited by Pichichero and colleagues.

The question of whether thiomersal increases the risk of neurodevelopmental disorders, as the Institute of Medicine's Immunization Safety Review Committee thought "biologically plausible", extends beyond childhood vaccines.[3] Until recently, the immune globulin (RhoGAM), which is given to rhesus-negative women one or more times during pregnancy or immediately postpartum, or both, contained thiomersal.[4] Organic mercury readily crosses the placenta, the blood-brain barrier, and is excreted in breast milk.[5]

Despite its use as a preservative in vaccines and other biological products for more than 60 years, the safety of thiomersal in infants has not been systematically studied. Future investigations, preclinical and clinical, need to define the pharmacokinetic profile and neurotoxic potential of thiomersal in the highest-risk populations: newborns and fetuses. Until such studies are done, every effort should be made to limit fetal and infant exposure to this mercury compound.

The above letter was written in the private capacity of the author, and the opinions expressed therein should not be construed as the official position of the US Food and Drug Administration.

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Sir—Michael Pichichero and colleagues[1] provide important new data on mercury concentrations in blood after thiomersal exposure.

The estimated half-life of ethylmercury (7 days) is shorter than that of methylmercury, and there is no evidence that ethylmercury exposure accumulates over time from repeat exposures to thiomersal. Pichichero and colleagues, and D C Henderson,[2] note that mercury concentrations in blood did not exceed 29 nmol/L—the safety level recommended by a panel of the US National Academy of Sciences for methylmercury.[3] However, the authors did not measure the peak blood concentrations that occurred within hours after the injections. If the true half-life of ethylmercury is 7 days, the mercury concentrations in blood measured 7 days after exposure are about half the peak concentrations, and blood concentrations measured 21 days after exposure are about an eighth of the peak concentrations.

Pichichero and colleagues should generate a model for exposures that took place at birth after the administration of hepatitis B vaccine and at 6–8 weeks of age when infants received up to 62·5 μg ethylmercury. Their model should estimate mercury concentrations in blood for infants who are at the 5th and 50th percentiles in bodyweight. For example, what would be the estimated concentrations for a 2·5 kg 6-week-old infant who received 62·5 μg mercury?

The data are available for one child in their study: a 2-month-old with a blood mercury concentration of 20·55 nmol/L 5 days after vaccination. If the ethylmercury half-life is 7 days (and half lives are variable), the peak mercury concentration in blood would have been 29·4 nmol/L, which is right at the safety level. This leaves no margin of safety if there were other sources of mercury exposure as well, belying the authors' claim that "no children had a concentration of blood mercury exceeding 29 nmol/L". This child reportedly received 37·5 μg mercury; a dose of 62·5 μg could well have resulted in a peak blood mercury concentration of 48·3 nmol/L. Their figure 1 indicates that there was a 2-month-old with 7 nmol/L mercury in blood at day 21, which would imply a possible peak blood mercury concentration of 42 nmol/L if the half-life is indeed 7 days.

The infants studied by Pichichero and colleagues seem to have come from a population with low background exposure to methylmercury. The ability to measure any mercury in blood from thiomersal exposures is of potential concern for infants born to mothers with high blood concentrations of methylmercury from fish consumption if the effects of ethylmercury are additive to those of methylmercury.[4] The US National Research Council has estimated that about 60 000 children are born in the USA every year to mothers who have concentrations of methylmercury in blood that put their infants at potential risk of harmful effects.[3] Hopefully someone is doing animal studies to determine whether exposure to ethylmercury is additive to that of methylmercury.

Additional data should soon become available from neurodevelopmental testing of children who were exposed to

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Review

Mercury as an environmental stimulus in the development of autoimmunity – A systematic review

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ABSTRACT

Autoimmune diseases result from an interplay of genetic predisposition and factors which stimulate the onset of disease. Mercury (Hg), a well-established toxicant, is an environmental factor reported to be linked with autoimmunity. Hg exists in several chemical forms and is encountered by humans in dental amalgams, certain vaccines, occupational exposure, atmospheric pollution and seafood. Several studies have investigated the effect of the various forms of Hg, including elemental (Hg^0), inorganic (iHg) and organic mercury (oHg) and their association with autoimmunity. *In vitro* studies using peripheral blood mononuclear cells (PBMC) from healthy participants have shown that methylmercury (MeHg) causes cell death at lower concentrations than iHg albeit exposure to iHg results in a more enhanced pro-inflammatory profile in comparison to MeHg. *In vivo* research utilising murine models susceptible to the development of metal-induced autoimmunity report that exposure to iHg results in a lupus-like syndrome, whilst mice exposed to MeHg develop autoimmunity without the formation of immune complexes. Furthermore, lower concentrations of IgE are detected in MeHg-treated animals in comparison with those treated with iHg. It appears that, oHg has a negative impact on animal models with existing autoimmunity. The research conducted on humans in this area is diverse in study design and the results are conflicting. There is currently no evidence to implicate a role for Hg^0 exposure from dental amalgams in the development or perpetuation of autoimmune disease, apart from some suggestion of individual sensitivity. Several studies have consistently shown a positive correlation between iHg exposure and serum autoantibody concentrations in gold miners, although the clinical impact of iHg remains unknown. Furthermore, a limited number of studies have reported individuals with autoimmune disease have higher concentrations of blood Hg compared to healthy controls. In summary, it appears that iHg perpetuates markers of autoimmunity to a greater extent than oHg, albeit the impact on clinical outcomes in humans is yet to be elucidated.

Contents

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Chronic Metals Ingestion By Prairie Voles Produces Sex-Specific Deficits In Social Behavior: An Animal Model Of Autism

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Abstract

We examined the effects of chronic metals ingestion on social behavior in the normally highly social prairie vole to test the hypothesis that metals may interact with central dopamine systems to produce the social withdrawal characteristic of autism. Relative to water-treated controls, ten weeks of chronic ingestion of either Hg^{++} or Cd^{++} via drinking water significantly reduced social contact by male voles when they were given a choice between isolation or contact with an unfamiliar same-sex conspecific. The effects of metals ingestion were specific to males: no effects of metals exposure were seen in females. Metals ingestion did not alter behavior of males allowed to choose between isolation or their familiar cagemates, rather than strangers. We also examined the possibility that metals ingestion affects central dopamine functioning by testing the voles' locomotor responses to peripheral administration of amphetamine. As with the social behavior, we found a sex-specific effect of metals on amphetamine responses. Males that consumed Hg^{++} did not increase their locomotor activity in response to amphetamine, whereas similarly-treated females and males that ingested only water significantly increased their locomotor activities. Thus, an ecologically relevant stimulus, metals ingestion, produced two of the hallmark characteristics of autism – social avoidance and a male-oriented bias. These results suggest that metals exposure may contribute to the development of autism, possibly by interacting with central dopamine function, and support the use of prairie voles as a model organism in which to study autism.

Keywords

microtus; autism; dopamine; toxicology; metal; mercury; social behavior; prairie vole

Introduction

The autism spectrum disorders are widespread in the developed world and the incidence of autism may be increasing. It is clear from several decades of study that autism is a complex (of) disorder(s) involving both genetic and environmental factors, but there is far from

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Chronic inorganic mercury exposure induces sex-specific changes in central TNFα expression: Importance in autism?

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Abstract

Mercury is neurotoxic and increasing evidence suggests that environmental exposure to mercury may contribute to neuropathologies including Alzheimer's disease and autism spectrum disorders. Mercury is known to disrupt immunocompetence in the periphery, however, little is known about the effects of mercury on neuroimmune signaling. Mercury-induced effects on central immune function are potentially very important given that mercury exposure and neuroinflammation both are implicated in certain neuropathologies (i.e., autism). Furthermore, mounting evidence points to the involvement of glial activation in autism. Therefore, we utilized an in vivo model to assess the effects of mercury exposure on neuroimmune signaling. In prairie voles, 10 week mercury exposure (60 ppm $HgCl_2$ in drinking water) resulted in a male-specific increase in TNFα protein expression in the cerebellum and hippocampus. These findings are consistent with our previously reported male-specific mercury-induced deficits in social behavior and further support a role for heavy metals exposure in neuropathologies such as autism. Subsequent studies should further evaluate the mechanism of action and biological consequences of heavy metals exposure. Additionally, these observations highlight the potential of neuroimmune markers in male voles as biomarkers of environmental mercury toxicity.

Keywords

heavy metals; environmental toxins; voles; cytokines; chemokines; autism

Introduction

Environmental exposure to heavy metals is a significant risk to human health [12]. Mercury, for example, certainly is neurotoxic and accumulation of mercury in the brain is accompanied by abnormal neuronal function in several brain regions, including in the cerebellum and the hippocampus [5, 17, 54]. Increasing evidence suggests that environmental mercury exposure may contribute to neuropathologies such as Alzheimer's disease (AD) and the autism spectrum disorders (ASD) [20-22, 28, 38-40].

Among the mechanisms implicated in mercury-induced neurotoxicity are mitochondrial dysfunction and oxidative stress [33, 45]. However, sub-lethal exposure to mercury also disrupts immunocompetence [29, 30] suggesting that changes in neuroimmune function may

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A brain proteome profile in rats exposed to methylmercury or thimerosal (ethylmercury)

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ABSTRACT

Exposure to organomercurials has been associated with harmful effects on the central nervous system (CNS). However, the mechanisms underlying organomercurial-mediated neurotoxic effects need to be elucidated. Exposure to toxic elements may promote cellular modifications such as alterations in protein synthesis in an attempt to protect tissues and organs from damage. In this context, the use of a "proteomic profile" is an important tool to identify potential early biomarkers or targets indicative of neurotoxicity. The aim of this study was to investigate potential modifications in rat cerebral cell proteome following exposure to methylmercury (MeHg) or ethylmercury (EtHg). For MeHg exposure, animals were administered by gavage daily 140 µg/kg/d of Hg (as MeHg) for 60 d and sacrificed 24 h after the last treatment. For EtHg exposure, 800 µg/kg/d of Hg (as EtHg) was given intramuscularly (im) in a single dose and rats were sacrificed after 4 h. Control groups received saline either by gavage or im. After extraction of proteins from whole brain samples and separation by two-dimensional electrophoresis (2-DE), 26 differentially expressed proteins were identified from exposed animals by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF/TOF). Both MeHg and EtHg exposure induced an overexpression of calbindin, a protein that acts as a neuroprotective agent by (1) adjusting the concentration of Ca^{2+} within cells and preventing neurodegenerative diseases and (2) decreasing expression of glutamine synthetase, a crucial protein involved in regulation of glutamate concentration in synaptic cleft. In contrast, expression of superoxide dismutase (SOD), a protein involved in antioxidant defense, was elevated in brain of MeHg-exposed animals. Taken together, our data provide new valuable information on the possible molecular mechanisms associated with MeHg- and EtHg-mediated toxicity in cerebral tissue. These observed protein alterations may be considered as biomarkers candidates for biological monitoring of organomercurial poisoning.

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Mercury (Hg), as element toxic to mammals and other animals, is found in the environment in three distinct chemical forms: as elemental or metallic Hg, as inorganic Hg, or as organic Hg. In addition, toxic properties are directly related to the chemical form of Hg (Bernhoft, 2012; Carneiro et al., 2014a, 2014b; Dorea et al., 2014). The most common forms of organomercurials are methylmercury (MetHg) and ethylmercury (EtHg) (thimerosal). Eating contaminated fish and shellfish is the main source of MetHg exposure (Sweet and Zelikoff, 2001). On the other hand, EtHg has been widely used as a preservative in a number of drug products, including vaccines, to help prevent life-threatening contamination with microbes (Tan and Parkin, 2000). Almost every human and animal (domestic and farmed) that has been immunized with thimerosal-containing vaccines has been exposed to EtHg (Dorea et al., 2013).

Exposure to organic forms of Hg is associated with several neurologic disorders, including cerebellar neurodegeneration, loss of cells from the granular layer, visual impairment with loss of cells of the cortex, peripheral nerve degeneration, and sensory disturbances (Clarkson et al., 2003; Clarkson and Magos, 2006; Counter et al., 2002; Eto et al., 2002; Grandjean and Herz, 2011; Johansson et al.,

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Blood Levels of Mercury Are Related to Diagnosis of Autism: A Reanalysis of an Important Data Set

M. Catherine DeSoto, PhD, and Robert T. Hitlan, PhD

The question of what is leading to the apparent increase in autism is of great importance. Like the link between aspirin and heart attack, even a small effect can have major health implications. If there is any link between autism and mercury, it is absolutely crucial that the first reports of the question are not falsely stating that no link occurs. We have reanalyzed the data set originally reported by Ip et al. in 2004 and have found that the original *p* value was in error and that a significant relation does exist between the blood levels of mercury and diagnosis of an autism spectrum disorder. Moreover, the hair sample analysis results offer some support for the idea that persons with autism may be less efficient and more variable at eliminating mercury from the blood.

Keywords: autism; mercury; environmental health; neurotoxin; neurodevelopment; blood

There is a marked increase in the diagnosis of autism. The question of what is (and is not) related to this increase is crucial to millions of persons affected by the disorder. This article reanalyzes an original data set regarding the relation between blood levels of mercury and diagnosis of an autism spectrum disorder (ASD) by Ip et al. based on our finding of discrepancies in the original article.[1]

A review of what is known about the neurotoxic effects of mercury is beyond the scope of this paper,[2] but the observable symptoms of acute mercury poisoning have been reported to match up with many of the problems observed in autism.[3] Furthermore, mercury poisoning has sometimes been presumptively diagnosed as autism of unknown etiology until the mercury poisoning has been uncovered.[4] Because there has been a several-fold increase in environmental mercury exposure, the hypothesis that the rise in autism could be related to an environmental increase in mercury levels is a reasonable one to pursue. Autism may result from a combination of genetic susceptibility (perhaps in the form of reduced ability to remove mercury or other neurotoxins from the system) and environmental exposure at key times in development.[5-7] This would mean a generalized increase in mercury levels would be expected to co-occur with a generalized increase in autism, but some people exposed to relatively high mercury would not be affected if, for example, their bodies were very efficient eliminators of such toxins. Only if an exposed infant or fetus also had a genetic susceptibility that makes one less able to remove mercury (or other heavy metals) would normal levels of mercury exposure lead to problems. Alternatively, it could be that genes that help detoxify get switched on and start to express themselves a little later than normal in those genetically predisposed to autism; or perhaps, autism results from some combination of these theories.

Nevertheless, if mercury does play any causal role in facilitating a diagnosis of autism, there would likely be at least some relation between high mercury measured in the blood and symptoms of autism even if ability to metabolize mediates the relationship between exposure and neural toxicity. This is because even if exposure is identical, those who remove mercury less effectively should still have higher levels in the blood. Interestingly, results of hair samples could be expected to be somewhat mixed. The level of mercury in hair may be better understood as an indication of how much mercury has been removed by the body as opposed to the level in the body.[6] If people are approximately equal in their ability to remove circulating mercury from the bloodstream, then these 2 indicators should match up closely, but if a person's ability to excrete is low, their hair samples might not be elevated even when their blood levels are high.

Fido and Al-Saad found that mercury levels in hair samples were higher in children diagnosed with autism.[8] These children were aged 4 to 7. In contrast, Kern et al. reported that mercury hair levels were not significantly different, but were lower at a marginally significant level.[9] Kern et al. used younger children, ages 1 to 6. Holmes et al. performed the

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Sorting out the spinning of autism: heavy metals and the question of incidence

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The reasons for the rise in autism prevalence are a subject of heated professional debate. Featuring a critical appraisal of some research used to question whether there is a rise in cases and if rising levels of autism are related to environmental exposure to toxins (Soden et al. 2007, Thompson et al. 2007, Barbaresi et al. 2009) we aim to evaluate the actual state of scientific knowledge. In addition, we surveyed the empirical research on the topic of autism and heavy metal toxins. Overall, the various causes that have led to the increase in autism diagnosis are likely multi-faceted, and understanding the causes is one of the most important health topics today. We argue that scientific research does not support rejecting the link between the neurodevelopmental disorder of autism and toxic exposures.

Key words: autism, autism prevalence, heavy metals, mercury, toxins

INTRODUCTION

In this paper, we argue that increasingly over the past decade, positions that deny a link to environmental toxins and autism are based on relatively weak science and are disregarding the bulk of scientific literature. In this paper, we are not focusing on vaccines, which is but one exposure pathway, but on exposure to toxic heavy metals as a broader class, of which a vaccine containing a heavy metal preservative would be but one possibility of exposure. It should be clear that any link between toxins and autism is almost certainly mediated by one's genetic makeup, and that other toxins, such as organophosphates (Eskenazi et al. 2007) likely play a role as well. In this conceptualization, the gene pool did not change, but exposure to substances that directly affect gene functioning is changing. Therefore, the reason why one five year old has developed autism and another has not, is indeed in large part a function of the individual's genes. But the question is still why more children are being diagnosed as autistic today than 30 years ago. Many factors are different today than a generation ago: autism awareness, exercise, diet, use of sunscreen and outdoor play, the amount of toxins in the environment – to name just a few. It is the authors' opinion that all of these things matter. Nevertheless, our interest is in the exposure to toxins, and in this paper to toxic heavy metals. Some prominent researchers still deny that there has been any actual increase in the cluster of behaviors that fall under the umbrella of autism spectrum disorders (ASD). For example, Roy Grinker in his top selling book on autism (2007) denies an actual increase has occurred, maintaining that it is all due to increased awareness and broadening of the diagnosis. Our opinion is not only that the increase is real, but that the increase in various contaminants is a major factor responsible for that increase.

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QUESTION OF THE RISE IN AUTISM INCIDENCE

Before further discussion, we wish to make clear the following: there is evidence for changes in diagnostic practice to have played a role in the autism prevalence rate. To our knowledge, there is no one who denies that diagnostic changes have occurred. When adherents to

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Review

How environmental and genetic factors combine to cause autism: A redox/methylation hypothesis

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Abstract

Recently higher rates of autism diagnosis suggest involvement of environmental factors in causing this developmental disorder, in concert with genetic risk factors. Autistic children exhibit evidence of oxidative stress and impaired methylation, which may reflect effects of toxic exposure on sulfur metabolism. We review the metabolic relationship between oxidative stress and methylation, with particular emphasis on adaptive responses that limit activity of cobalamin and folate-dependent methionine synthase. Methionine synthase activity is required for dopamine-stimulated phospholipid methylation, a unique membrane-delimited signaling process mediated by the D4 dopamine receptor that promotes neuronal synchronization and attention, and synchrony is impaired in autism. Genetic polymorphisms adversely affecting sulfur metabolism, methylation, detoxification, dopamine signaling and the formation of neuronal networks occur more frequently in autistic subjects. On the basis of these observations, a ''redox/methylation hypothesis of autism'' is described, in which oxidative stress, initiated by environment factors in genetically vulnerable individuals, leads to impaired methylation and neurological deficits secondary to reductions in the capacity for synchronizing neural networks.

Keywords: Arsenic; Attention; Attention-deficit hyperactivity disorder (ADHD); D4 dopamine receptor; Folic acid; Heavy metal; Lead; Mercury; Oxidative stress; Neuronal synchronization; Pesticide; Phospholipid methylation; Thimerosal; Vitamin B_{12}; Xenobiotic

Contents

During the past several decades the prevalence of autism and related pervasive developmental disorders in the U.S. has dramatically escalated to epidemic levels, affecting 3 in 10,000 children in 1970, but 66 in 10,000 in 2002 (Rice et al., 2007). The possible origins of this increase have been the subject of considerable public debate (Blaxill, 2004), and advances in detection and broadening of the diagnostic criteria for autism have been suggested to play a role (Fombonne et al., 2006), while genetic factors are clearly important, as indicated by high concordance rates among twins and siblings (Smalley et al., 1988). However, genetic factors alone cannot account for an epidemic that developed in the relatively short period of 10–20

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Autism spectrum disorder prevalence and associations with air concentrations of lead, mercury, and arsenic

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Abstract Lead, mercury, and arsenic are neurotoxicants with known effects on neurodevelopment. Autism spectrum disorder (ASD) is a neurodevelopmental disorder apparent by early childhood. Using data on 4486 children with ASD residing in 2489 census tracts in five sites of the Centers for Disease Control and Prevention's Autism and Developmental Disabilities Monitoring (ADDM) Network, we used multi-level negative binomial models toinvestigate if ambient lead, mercury, and arsenic concen-trations, as measured by the US Environmental Protection Agency National-Scale Air Toxics Assessment (EPA-NATA), were associated with ASD prevalence. In unad-justed analyses, ambient metal concentrations were nega-tively associated with ASD prevalence. After adjusting for confounding factors, tracts with air concentrations of lead in the highest quartile had significantly higher ASD prev-alence than tracts with lead concentrations in the lowest quartile (prevalence ratio (PR) = 1.36; 95 '% CI: 1.18, 1.57). In addition, tracts with mercury concentrations above the 75th percentile (>1.7 ng/m3) and arsenic con-centrations below the 75th percentile (≤0.13 ng/m3)hada significantly higher ASD prevalence (adjusted RR = 1.20; 95 % CI: 1.03, 1.40) compared to tracts with arsenic, lead, and mercury concentrations below the 75th percentile. Our results suggest a possible association between ambient lead concentrations and ASD prevalence and demonstrate that exposure to multiple metals may have synergistic effects on ASD prevalence.

Keywords Metals . Autism spectrum disorder . Environment . Pollution . Air quality

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Introduction

Autism spectrum disorder

Autism spectrum disorder (ASD) is a developmental disorder characterized by impairments in social interaction, communication, and behavior evident in early development. According to the 2014 surveillance estimate from the Centers for Disease Control and Prevention (CDC), the prevalence of ASD in the USA may be approximately 1 in 45 (Zablotsky et al. 2015). To date, the etiology of ASD has been poorly defined; however, some studies have suggested that ASD may be caused by interactions of susceptible genes with the environment in which environmental triggers may alter gene expression (Volk et al. 2014; Blake et al. 2013; LaSalle 2013; Herbert et al. 2006). Therefore, several investigators have examined the relationships between ASD and exposures to pesticides (Shelton et al. 2012;

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Hair mercury concentrations in Korean infants could be influenced by thimerosal-containing vaccines

Dear Editor,

The paper by Kim et al. (2008) determining hair-Hg in Korean children (median age of 18 months) clearly included young infants (<6 months). They detailed their work according to levels of hair-Hg concentrations and explained children's Hg exposure through fish consumption. While hair-Hg is an acceptable indicator of fish-methylmercury exposure, there is a chance that infants' hair-Hg in their study may also contain Hg-metabolites as a result of immunization using thimerosal-containing vaccines (TCV).

Over the last 20 years there has been an increase in the number of TCV given to infants (Dórea, 2007) and one of these vaccines, hepatitis B, is given to newborns in the first day of life (Marques et al., 2007b). Given the age of the infants studied by Kim et al., it is possible that the hair samples of the very young ones may have contained not only TCV-Hg, but also ethylmercury (EtHg) derived from mothers using products containing thimerosal during pregnancy; Rh-negative mothers could have taken anti-RhoD immune globulins and may have passed some EtHg to the newborn.

Although TCV is no longer used in the USA and developed nations, it is still used in a score of other countries. Additionally, because the Hg-preservative (thimerosal) in vaccines vary according to product manufacturer, immunized infants are exposed to varied EtHg concentrations (depending on the vaccine maker) and a wide range of doses — depending on the infant's weight (Dórea and Marques, 2008). As a consequence, additional TCV-EtHg exposure may contribute to a relative increase in infant's hair-Hg (Marques et al., 2007a). In such circumstances the reader needs to know the occurrence of pre- and post-natal EtHg exposure. Although Kim et al. referred to a recent paper dealing with different sources of Hg exposure and children's hair-Hg levels (Marques et al., 2007b), they did not inform on the type of vaccines (used in young children) or thimerosal products (used by mothers during pregnancy) that might contribute to the levels of hair-Hg concentrations. However, we were informed that participants were recruited during immunization visits to health centers (Kim et al., 2008). Therefore, the contribution of Kim et al. could be enhanced with a post-hoc discussion of this issue.

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LETTER TO THE EDITOR

Modeling Neurodevelopment Outcomes and Ethylmercury Exposure from Thimerosal-Containing Vaccines

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Dear Editor,

The neurotoxic effects of ethylmercury (EtHg) accidentally consumed in Iraq were sufficient to withdraw ethylmercury-containing fungicides as seed dressing. Despite that, not only did thimerosal continue to be used in pharmaceutical preparations but also toxicological interest in EtHg-derived substances diminished considerably and was never addressed with regard to the small quantities used as a vaccine preservative. Thimerosal-containing vaccines (TCV) have no record of overt clinical neurological consequences due to EtHg, and the plausibility of subtle neurotoxic effects in children has been recognized only recently by the United States and other industrialized countries. In this context, we welcome the interesting work of Berman *et al.* (2008); it is clear that this assiduous study (in immunologically susceptible mice) took into consideration doses and schedules of TCV-Hg concentrations that had been used in infants in the United States. Their mice model does not, however, cover the full extent of modifying factors associated with TCV-Hg exposure in the majority of immature and newborns around the world that still have to depend on TCV.

According to Berman *et al.* (2008), the United States vaccination scheduled exposed a total of 125 μgHg distributed at 2, 2, and 6 months through TCV (hepatitis B and DTP). This type of vaccine is no longer used in industrialized countries but it is still used all over the world. We know that thimerosal concentrations vary among brands of vaccines and also that immunization schedules vary depending on a country's health policy; not only that but new outbreaks of disease introduce additional new vaccines (which may contain thimerosal) during the first year of life. As an example, the public health services of Brazil, like other countries, still uses several brands of hepatitis B vaccine (containing thimerosal as preservative) with concentrations ranging from 12.5 to 50 μgHg per 0.5 ml shot. Another salient difference between countries that use TCV (like Brazil) and the United States is that in the former country hepatitis B inoculation starts within the first 12–24 h after birth (Marques *et al.*, 2007) and is administered to low-birth weight $\geq$2000 g (Ministério, da Saúde, 2006 and premature babies who are also recommended a fourth shot as an additional booster (DI/DH/CVE, 2006). In such situations, not only toxicokinetics (TK) but especially toxicodynamics (TD) of EtHg are entirely different between a 1-day-old (with different stages of immaturity and birth weight) and a 60-day-old child (as modeled).

The newborn presents several physiological degrees of immaturity in the excretory system (kidneys and bile formation) and target organ (central nervous system, CNS) that are important modifiers of EtHg TK and TD. These features are inversely accentuated by gestational age and birth weight. Under such circumstances, unbound circulating EtHg in a newborn (and immature) may not be eliminated as fast as in a 2-month-old baby and thus will be readier to cross the more vulnerable blood-brain barrier (BBB). The newborn BBB increases in effectiveness with age; therefore, the free EtHg can more easily penetrate the immature CNS (Dorea, 2007). As a consequence, the smaller the body size and blood volume, the more altered the TD and TK of EtHg. Indeed, Stajich *et al.* (2000) showed that preterm infants do not metabolize Hg efficiently. Collectively, studies show that larger babies have significantly higher mean liver metallothionein than smaller babies (Dorea, 2007).

Factors associated with protein-binding capacity, excretion mechanisms, and enzyme activities are immature in the neonate and modulate differences in adverse effects between newborns and infants exposed to neurotoxic substances. During the period of immaturity, not only plasma albumin but also total protein concentrations decrease (Dorea, 2007). The best example in differences between neurotoxic effects is the type of albumin and competition for binding sites (due to increased circulatory concentrations of bilirubin). Albumin binding (to bilirubin) is less effective during the first postnatal days and, as a consequence, excess free bilirubin can cross the BBB at early stages of the postnatal CNS immaturity

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and cause brainstem abnormalities; albumin priming can be effective in attenuating effects caused by unbound bilirubin (Dorea, 2007).

We do not dispute the conclusions drawn by Berman *et al*. regarding Hg and the neurobiology of autism; however, we think it is possible to take their findings one step further in regards to thimerosal neurotoxicity. We contend that these findings are appropriate for U.S.-like scenarios (as intended by the authors) but are not sufficient to address the current TCV schedules in the majority of newborns and infants around the world. TCV are used worldwide in vaccination schedules that include more of these vaccines at an earlier age. Unfortunately, the differences that set newborns (especially low-birth-weights and prematures) apart from 2-month-old infants have not yet been modeled in experimental studies and remain neglected in TK and TD knowledge of TCV-EtHg exposure. We hope that studies like Berman *et al*. (2008) can inspire conventional toxicology to address uncertainties regarding current serial EtHg exposure in newborns and infants that have to take TCV.

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NeuroToxicology

Letter to the Editor

Comparing fish-mercury exposed Amazonian children: Should not we consider thimerosal-preserved vaccines?

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Two outstanding research groups pooled data in a unique study comparing French Guiana and Brazil regarding subtle (subclinical) neurotoxic effects of Hg exposure attributed to fish consumption (Chevrier et al., 2008): there were increased risks of making rotation or simplification errors in the drawings by children with an increased level of Hg. The Brazilian children of Rio Tapajos (Sai Cinza, Brasilia Legal, and Santana do Ituquí) had twice the mean levels of hair-Hg concentrations compared with the Wayama children of French Guiana; thus indicating a higher mean fish intake.

We fully agree with this basic assumption, but, when assuming sources of Hg exposure there could be other differences between the two countries (French Guiana and Brazil) regarding organic-mercury, such as ethylmercury derived from thimerosal-containing vaccines (TCVs). Although "all the children were chemically exposed to meHg during gestation and into childhood" there is a strong possibility that the Brazilian children could be additionally exposed to ethylmercury, both during gestation and infancy, as a result of serial injections of TCV. Additionally, there are other environmental factors that act as effect modifiers of neurocognitive outcomes.

Brazil has an efficient immunization program using TCVs in young children (Dórea, 2007). Moreover, vaccines against diphtheria and tetanus (DT) are also part of pre-natal care widely available for mothers in Brazil starting at the second month of pregnancy. After birth, both hepatitis B and DTP vaccines are given to young children (Dórea, 2007). In Brazil all these vaccines are preserved with thimerosal at concentrations that depend on the vaccine manufacturer (Dorea and Marques, 2008). According to the Brazilian immunization schedule, a 6-month-old infant can receive six shots (three hepatitis B, and three DTP) of TCVs. Coupled with maternal TCV taken during pregnancy, this substantial Hg load (in the fetus and in infancy) occurs at critical windows of central nervous system vulnerability.

Unlike Brazil, French Guiana is an oversea territory of France, and Amazonian children there are probably vaccinated with thimerosal-free vaccines. Indeed, in France, according to Freed et al. (2002), "the first dose of the hepatitis B vaccine is recommended at 2 months of age, not at birth, for children born to mothers whose hepatitis B surface antigen status is negative. The only vaccines in France containing thimerosal are the hepatitis B and influenza vaccines, and there were always thimerosal-free options for these vaccines. Therefore, a thimerosal-free hepatitis B vaccine was available for at-risk infants who received the hepatitis B vaccine at birth." In Brazil, neonates take thimerosal-preserved hepatitis-B vaccine within the first day after birth with a wide range of TCV-Hg doses (10 times), depending on birth weight and the vaccine manufacturer (Dórea and Marques, 2008).

Although the small dose of thimerosal in vaccines is considered safe, the recent work of Gallagher and Goodman (2008) showed an increased risk from hepatitis B vaccination in American children associated with special educational needs. In the context of fish-Hg exposure of Amazonian children, the ethylmercury load is never taken into consideration. Instead, the issue of proximity to alluvial gold extractuin has always taken precedence; while TCV-Hg exposure is universal in the Brazilian Amazon gold mining is only relevant for families occupationally engaged in gold amalgamation activities.

It is well documented that malaria is endemic in most of the Amazon forest, regardless of country boundaries. In Brazil, there is information pertaining not only to malaria, but also to intestinal parasites in the children of the Munduruku villages and other *ribeirinhos*; malaria affects 100% of the adult population and presumably a relative proportion of their children. Besides the differences in overall nutritional status between children of the two countries we were also not informed of differences in malaria incidence between French Guianan and Brazilian communities. Some of the sample of the children from the French Guiana dataset was from the Galipi community living in Awala on the Atlantic coast.

Indirectly, malaria can cause iron deficiency, and malnutrition secondary to intestinal parasites can slow intelligence development in children. In this regard, we compared *ribeirinho* children of the Amazon with agrarian children (non-fish consumers) and found very minor differences in psychometric tests. In these isolated communities there was a disparity of mean hair concentrations (66 times higher in *ribeirinhos*) and only minor differences in the poor performance of both groups (Fonseca et al., 2008). Therefore we agree with Chevrier et al. that for neurocognitive-performance tests there could be determinants other than fish-MeHg exposure.

Regardless of confounders and environmental differences, Chevrier et al.'s work remains an important contribution to realizing that cognitive development may take longer in the more Hg-exposed group resulting in unpredictable consequences. My comments are meant to stimulate further research on all aspects of Hg exposure that might affect neurocog-

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Neonate Exposure to Thimerosal Mercury from Hepatitis B Vaccines

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ABSTRACT

Infant exposure to ethylmercury (EtHg) has not only increased but is starting earlier as a result of the current immunization schedule that uses thimerosal-containing vaccines (TCVs). Although vaccination schedule varies considerably between countries, infants in less-developed countries continue to be exposed to EtHg derived from more affordable TCVs. We studied the exposure of newborns to EtHg from hepatitis B vaccines; hospital records (21,685) were summarized for the years 2001 to 2005 regarding date of birth, vaccination date, and birth weight. Most of the vaccinations occurred in the first 24 hours postdelivery; over the 5 years, there was an increase in vaccinations within hours of birth (same day), from 7.4% (2001) to 87.8% (2005). Nearly 94.6% of infants are now being vaccinated within the first 24hours. Range of mercury exposure spread from 4.2 to 21.1 μg mercury/kg body weight for those receiving TCVs with the highest thimerosal concentration; these exposure levels are conservative for 2% of children receiving vaccines within 2 to 3 postnatal days, when they are still going through physiological postnatal weight loss. Because of the particular timing (transitioning from in utero to ex utero metabolism) and specific aspects of exposure (i.e., parenteral mode, bypassing gastroenteric barriers) and dose (related to vaccine manufacturer and with variation in birth weight), this study reveals critical issues that can modulate toxicokinetics and toxicodynamics of organomercurials in neonates.

KEYWORDS: Thimerosal, hepatitis B, ethylmercury, newborns, immunization

Mercury (Hg) is a widely recognized neurotoxic element, and fetus and infants are especially vulnerable to its effects. The developing brains of newborns are susceptible to all forms of Hg, and thimerosal in vaccines is still the first line of exposure to infants in less-developed countries.[1] Because of differences in vaccination policies, the overall exposure to thimerosal has always varied between countries.[2] Despite low doses in vaccines, it is plausible that fetuses and young infants may be susceptible to untoward effects of thimerosal.[2] Although the United States and the EU countries reduced or eliminated thimerosal from most vaccines and immunoglobulins in 1999, thimerosal-containing vaccines (TCVs) continued to be used in less-developed and developing countries. However, still in the United States, it is recommended that all pregnant women, infants, and children (until 18 years old) receive annual influenza vaccination, of which more than 90% still contain thimerosal. Because of the low concentration of thimerosal used as preservative, the World Health Organization considers it safe in TCVs.

Animal studies have established differential neurotoxicity of ethylmercury (EtHg) and methylmercury (MeHg).[3,4] However, public health concern about

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Commentary

Exposure to low-dose mercury (from thimerosal) & premature puberty - A new avenue for research with the vaccine safety datalink

The paper by Geier *et al*[1] addresses the plausible association of premature puberty after a typical pattern of exposure to ethylmercury in thimerosal-containing vaccines (TCVs) taken by young children in the USA before TCVs were discontinued. Both precocious puberty and low-level mercury are *per se* high-profile topics of public health interest. Given that TCVs are still currently given to pregnant women, infants and young children around the world, the paper raises a unique opportunity for discussing the role of mercury-based preservatives.

The study took advantage of the vaccine-safety datalink (VSD) system of the USA. Black *et al*[2] summarized the advantage of the VSD over the former Vaccine Adverse Event Reporting System (VAERS) in use until 1991 in the USA. Until then, potential vaccine safety issues could only be evaluated by the passive data collected through the VAERS. The current VSD system links outcome and vaccine exposure information, demographic and other covariate information, from the automated clinical databases within several Health Maintenance Organizations (HMOs). As pointed out by Black *et al*[2] this data bank can be utilized to screen for possible associations of events after vaccination and also, as in the case of Geier *et al*[1], to evaluate hypotheses. Geier *et al*[1] analyzed the data from 1990 to 1996 (n = 278,624) and explored a possible link of premature puberty to TCV received at young ages by comparing this outcome to outcomes not related to mercury exposure (controls). It is worth mentioning the disproportionate percentage of males (7%) in the sample. If encountered in future studies, this information confirms gender differences in thimerosal toxicity[3]. Constitutional differences in gender determine hormonal balance and represent a biologic variable[4] to be considered in reproductive and neurologic outcomes.

Premature sexual development is a topic of current interest because of social and attendant health-associated issues, especially for girls. Unwanted teenage pregnancy and sexually transmitted diseases are among the important social and biological issues affecting poor countries and disadvantaged segments of rich countries. Reports from different parts of the world indicate that precocious gynaecological-age is significantly associated with early sexual initiation[5] and with teenage pregnancy[6,7]. Additionally, as reviewed by Karaolis-Danckert *et al*[8], an accelerated age of puberty onset may influence the life-time risk for breast and testicular cancer, insulin resistance, and adiposity. It is becoming clear that environmental factors are strongly associated with precocious puberty[9]. Studies indicate that increasing rates of precocious puberty are among the endocrine-system related effects of endocrine-disruptor chemicals found in the environment[10].

Generally described as endocrine disruptors, there are a broad range of these substances capable of affecting the endocrine system. Some of these can act specifically on the reproductive system having estrogenic, anti-estrogenic, androgenic, and anti-androgenic activity. Besides that, these chemicals can also interfere with the hypthalamo-pituitary unit, and also disrupt estrous cyclicity. The endocrine-disrupting activity of these pollutants on developmental toxicology depends on timing and dosage. However, since these occur as mixtures, it is not yet possible to know if their end-point effects are additive or antagonistic. Therefore, this type of exposure is difficult to study because of the variety of possible outcomes[10]. A wide range of endocrine disruptors listed by Abaci *et al*[18] include biocides (herbicides, fungicides, insecticides, nematocides), and industrial compounds made up of organic substances and metals (that includes mercury).

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Infants' exposure to aluminum from vaccines and breast milk during the first 6 months

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The success of vaccination programs in reducing and eliminating infectious diseases has contributed to an ever-increasing number of vaccines given at earlier ages (newborns and infants). Exposure to low levels of environmental toxic substances (including metals) at an early age raises plausible concerns over increasingly lower neuro-cognitive rates. Current immunization schedules with vaccines containing aluminum (as adjuvant) are given to infants, but thimerosal (as preservative) is found mostly in vaccines used in non-industrialized countries. Exclusively, breastfed infants (in Brazil) receiving a full recommended schedule of immunizations showed an exceedingly high exposure of Al (225 to 1750 μg per dose) when compared with estimated levels absorbed from breast milk (2.0 μg). This study does not dispute the safety of vaccines but reinforces the need to study long-term effects of early exposure to neuro-toxic substances on the developing brain. Pragmatic vaccine safety needs to embrace conventional toxicology, addressing especial characteristics of unborn fetuses, neonates and infants exposed to low levels of aluminum, and ethylmercury traditionally considered innocuous to the central nervous system.
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Keywords: *aluminum, ethylmercury, thimerosal, breast milk, infants, adjuvant, vaccine.*

Introduction

Although vaccine safety is constantly reaffirmed in regard to its immunogenicity and rare adverse events, it is assumed that low doses of preservative (thimerosal) and adjuvant (aluminum salts) have the same innocuous effects across the large spectrum of those vaccinated — adults, children, infants, newborns, and unborn fetuses — and for the ever-increasing number of them given to young children. Despite low doses in vaccines, both Hg and Al are neuro-toxic; the higher toxicity of Hg is well recognized and it has been more studied and better understood than Al.

During early life, exposure to either mercury or aluminum that occur through breastfeeding depends on the maternal exposure (diet mainly). However, because of mammary-gland barrier, expected exposure for infants is greatly attenuated. The exposure to mercury or aluminum in breast milk is spread out through the course of a day's nursing with the very young or smaller (immature) baby absorbing proportionally smaller quantities. However, in intramuscular injections ethylmercury (in preservatives) and Al (as adjuvant) gain unimpeded access to body compartments. In this context, specific aspects of Hg exposure have been discussed elsewhere (Dórea, 2007). The American Academy of Pediatrics' revision of 1996 discussed aluminum in infant feeding but did not address the additional higher and acute exposure to aluminum in commonly used infants' vaccines (AAP, 1996).

Recent evidence based on cellular and animal studies indicates that both thimerosal at small concentrations (Baskin et al., 2003; Hornig et al., 2004; Ueha-Ishibashi et al., 2004; James et al., 2005; Parran et al., 2005; Geier et al., 2009; Hewitson et al., 2009; Olczak et al., 2009) and adjuvant-Al are neuro-toxic. In this regard, aluminum-adsorbed vaccines caused a transient rise in brain tissue of mice (Redhead et al., 1992). Indeed, *in vitro* work showed that adjuvant-Al at levels comparable to those administered to adults can kill motor neurons (Petrik et al., 2007). Toimela and Tähti (2004) showed the toxicity of both Al and Hg in neuro-blastoma cell line. The toxicity of Al is much lower than that of thimerosal (Deth et al., 2008). Nevertheless, Mutter et al. (2007) suggested that low levels of Hg could cause nerve cell deteriorations that could be aggravated by aluminum. Therefore, data to provide a non-observable adverse effect level for Hg and Al (inclusive combined) on the brain are sorely needed.

Vaccines represent an important strategic line of defense against infectious diseases; however, those containing mercurial

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Invited critical review

Making sense of epidemiological studies of young children exposed to thimerosal in vaccines

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ABSTRACT

Objective: To compare epidemiological studies dealing with neurological issues (compatible with Hg toxicity) linked to exposing newborns and infants to intramuscular doses of preservative-Hg resulting from vaccination with thimerosal-containing vaccines (TCV).
Methods: Major databases were searched for studies that addressed neurodevelopment outcomes other than autism. Eight studies were identified and compared.
Results: Information extracted from the studies done in the USA, the UK, and Italy is important in understanding the complex interplay of variables but insufficient to establish non-toxicity for infants and young children still receiving TCV: a) there is ambiguity in some studies reporting neurodevelopment outcomes that seem to depend on confounding variables; b) the risk of neurotoxicity due to low doses of thimerosal is plausible at least for susceptible infants; c) there is a need to address these issues in less developed countries still using TCV in pregnant mothers, newborns, and young children.
Conclusions: Since the use of TCV is still inevitable in many countries, this increases the need to protect vulnerable infants and promote actions that strengthen neurodevelopment. Developing countries should intensify campaigns that include breastfeeding among efforts to help prime the central nervous system to tolerate exposure to neurotoxic substances, especially thimerosal-Hg.

Contents

1. Introduction

It is estimated that 3% of neurodevelopmental disabilities (NDD) are directly linked to environmental neurotoxic substances and that 25% of these disabilities may arise as a result of interaction with individual genetic susceptibilities [1]. Outside a handful of rich countries, organic mercury (Hg) in the form of ethylmercury (EtHg) may be the first exposure a vaccinated infant has to a potentially neurotoxic substance such as mercury. EtHg is the metabolite of thimerosal widely used to preserve immunoglobulins (used by Rh-negative pregnant women) and vaccines that are given to pregnant mothers, newborns, infants, and young children.

Because the child is healthy when he/she takes vaccines, adverse events caused by vaccination are monitored to insure vaccine safety. Neurological syndromes and diseases may appear as a result of vaccine's antigens [2–4]; however, in the case of vaccine-thimerosal

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OVERVIEW

Integrating Experimental (In Vitro and In Vivo) Neurotoxicity Studies of Low-dose Thimerosal Relevant to Vaccines

José G. Dórea

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Abstract There is a need to interpret neurotoxic studies to help deal with uncertainties surrounding pregnant mothers, newborns and young children who must receive repeated doses of Thimerosal-containing vaccines (TCVs). This review integrates information derived from emerging experimental studies (in vitro and in vivo) of low-dose Thimerosal (sodium ethyl mercury thiosalicylate). Major databases (PubMed and Web-of-science) were searched for in vitro and in vivo experimental studies that addressed the effects of low-dose Thimerosal (or ethylmercury) on neural tissues and animal behaviour. Information extracted from studies indicates that: (a) activity of low doses of Thimerosal against isolated human and animal brain cells was found in all studies and is consistent with Hg neurotoxicity; (b) the neurotoxic effect of ethylmercury has not been studied with co-occurring adjuvant-Al in TCVs; (c) animal studies have shown that exposure to Thimerosal-Hg can lead to accumulation of inorganic Hg in brain, and that (d) doses relevant to TCV exposure possess the potential to affect human neuro-development. Thimerosal at concentrations relevant for infants' exposure (in vaccines) is toxic to cultured human-brain cells and to laboratory animals. The persisting use of TCV (in developing countries) is counterintuitive to global efforts to lower Hg exposure and to ban Hg in medical products; its continued use in TCV requires evaluation of a sufficiently nontoxic level of ethylmercury compatible with repeated exposure (co-occurring with adjuvant-Al) during early life.

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Keywords Children · Infants · Neurodevelopment · Pregnancy · Ethylmercury · Thimerosal

Introduction

The prevalence of emerging neuro-developmental disabilities has been directly linked to environmental neurotoxic substances which are estimated to affect 3% of children [1]; environmental mercury exposure, mainly methylmercury from seafood [1] and elemental mercury from coal combustion (used in electrical utilities) as well as municipal and medical waste incinerators [2], is at the center of concerns. However, a considerable part of these disabilities (25%) may arise as a result of interaction with individual genetic susceptibilities [1]. Indeed it is known that Hg neurotoxicity involves long latencies and atypical responses between low and high doses [3]; additionally, it has now been shown that exposure to different forms of mercury (such as methylmercury and Hg vapor) can act synergistically in increasing neurotoxic risks [3].

Organic and inorganic forms of mercury have a long history of use in medicine and pediatrics. Until the 1950s mercury preparations were part of the therapeutic resources to deal with common childhood ailments [4]. Because of its role in pink disease and also with the advent of more specific therapeutic drugs, mercury formulations have been withdrawn from children's medication [4]. Nevertheless, Thimerosal (sodium ethyl mercury thiosalicylate) has remained in wide use as a preservative in pharmaceutical products. Thimerosal in topical formulations has been eliminated in many parts of the world but its use in vaccines for pregnant women, newborns and young children continues in developing countries [5]. Although breast-fed infants can be exposed to elemental Hg from maternal

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Multiple toxic heavy metals and neonatal neurobehavior in China require considering co-exposure to Thimerosal-ethylmercury and adjuvant-aluminum

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The excellent study by Yu et al. (2011) is among the few that have examined the effects of mixed metal exposures in human infants. Two of Yu et al.'s objectives were "to determine the concentrations of key toxic heavy metals in umbilical cord blood and the corresponding effects on neurobehavioral development," and "to identify risk factors for prenatal toxic metal exposure." It is therefore crucial that we discuss all neurotoxic metals that infants are exposed to in utero as well as neonatally.

Yu et al. (2011) used a questionnaire to determine the fetus' potential exposure to toxic metals, including housing environment, parent's diet, smoking habits, and alcohol consumption. They paid particular attention to the amount and type of fish consumed by the mother. Nevertheless, there was no investigation into a prevalent source of organic mercury exposure in China — ethylmercury (EtHg) from Thimerosal-containing vaccines (TCV). To guarantee that the neurologic outcome data are validly associated with the neurologic insults, all neurotoxicants – including TCVs – must be considered.

In China, almost all neonates are given a dose of hepatitis-B vaccine within 24 h after birth. These TCVs may contain 7.5 to 17 μg ethylmercury (Gao et al., 2008); these vaccines are also adjuvanted with Al salts, which pose an additional load of another neurotoxic metal (Dórea and Marques, 2010). Pichichero et al. (2008) reported that 12 h after administration of such vaccines in newborn infants, there is a rise in blood Hg concentrations above the safe values of 5 ng/mL. Additionally, it is not uncommon for the vaccinated infant to develop fever and/or anorectic responses as a reaction to the immunologic stimuli of vaccination. For example, immediately after hepatitis B vaccination, Eales (2003) described behavioral changes in infants as "irritable and disinclined to feed." Additionally, there is enough experimental evidence (Dórea, 2011) and observational studies (Dórea, 2010) to base a reasonable concern that Thimerosal and Al (Tomljenovic and Shaw, 2011) in vaccines can affect young (susceptible) children.

So far, studies examining the possible untoward effects of ethylmercury or aluminum (from TCVs) have only modeled a single exposure (Dórea, 2010, 2011). The effects of both Hg and Al in vaccines need to be sufficiently studied to demonstrate that the combination of the two metals cause no harm. Improving our understanding of maternal/infant exposure to a combination of environmental and iatrogenic chemicals and its association with neurodevelopment is even more essential. As shown by Yu et al. (2011), it is axiomatic that early life exposure to toxic metals will lead to important neuronal insults. The regulation of TCVs in young children must then be based on evidence, and studies like Yu et al. (2011) carry the potential to address such issues.

Conflict of interest statement

Nothing declared.

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Low-Dose Mercury Exposure in Early Life: Relevance of Thimerosal to Fetuses, Newborns and Infants

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Abstract: This review explores the different aspects of constitutional factors in early life that modulate toxicokinetics and toxicodynamics of low-dose mercury resulting from acute ethylmercury (etHg) exposure in Thimerosal-containing vaccines (TCV).

Major databases were searched for human and experimental studies that addressed issues related to early life exposure to TCV. It can be concluded that: a) mercury load in fetuses, neonates, and infants resulting from TCVs remains in blood of neonates and infants at sufficient concentration and for enough time to penetrate the brain and to exert a neurologic impact and a probable influence on neurodevelopment of susceptible infants; b) etHg metabolism related to neurodevelopmental delays has been demonstrated experimentally and observed in population studies; c) unlike chronic Hg exposure during pregnancy, neurodevelopmental effects caused by acute (repeated/cumulative) early life exposure to TCV-etHg remain unrecognized; and d) the uncertainty surrounding low-dose toxicity of etHg is challenging but recent evidence indicates that avoiding cumulative insults by alkyl-mercury forms (which include Thimerosal) is warranted. It is important to a) maintain trust in vaccines while reinforcing current public health policies to abate mercury exposure in infancy; b) generally support WHO policies that recommend vaccination to prevent and control existing and impending infectious diseases; and c) not confuse the 'need' to use a specific 'product' (TCV) by accepting as 'innocuous' (or without consequences) the presence of a proven 'toxic alkyl-mercury' (etHg) at levels that have not been proven to be toxicologically safe.

Keywords: Thimerosal, ethylmercury, methylmercury, hair, blood, stools, vaccines, newborns, neurodevelopment.

1. INTRODUCTION

For certain infectious diseases, vaccines have been developed for the control and eradication of infectious diseases in susceptible populations. Before vaccine formulations are licensed, it is necessary to ensure that each is safe, efficacious and well tolerated *per se*. Today, the pertinent studies focus on the formulation's immunogenic components. For preservatives and adjuvants in vaccine formulations, a specific testing protocol for their safety and tolerability is rarely executed for their specific effects [1], particularly for Thimerosal-containing vaccines (TCVs) used in newborns and infants.

In the body, Thimerosal (used as a preservative in some vaccines) degrades into ethylmercury (etHg) chloride. After more than 60 years of use, concerns were raised regarding children's exposure to TCV-derived etHg [2]. Nevertheless, organic mercury fungicides have been banned in industrialized countries since the early 1970s [3]. The accidental consumption of organomercurial fungicides (which included etHg compounds) in fungicide-treated grains caused serious health problems in many parts of the world. These incidents and their adverse effects on humans, domesticated animals, and wild-life moved industrialized countries to ban these fungicides. As a result of these disastrous events, research emerged demonstrating how fast etHg can penetrate the brain of monkeys and rats [4]. Modeling oral exposure to high doses of Hg, the comparative toxicology of the two most studied forms of organic mercury (etHg and meHg) showed that, at high equimolar doses in gavage-treated rats, etHg was systematically more toxic than meHg (significant differences in weight loss and renal damage); however, in neurologic tests, few differences were reported between etHg and meHg with regard to their dorsal root ganglia or coordination disorders [5].

Despite the lack of specific studies addressing low doses of etHg in TCVs for young children, actions have been taken based on existing toxicological studies of etHg. In the last 15 years, recognizing the plausibility of low-dose etHg toxicity, countries in North America and the EU started to withdraw Thimerosal from vaccines for young children. It was only after reducing or withdrawing Thimerosal from these vaccines that studies appeared addressing children's neurodevelopment [6] and the toxicology of small doses relevant to vaccines [7].

The objective of this review is to present, to pediatricians and public health workers, the large body of evidence of adverse biological (mainly neurological) effects pertaining to small-dose etHg exposures at levels relevant to those in vaccines administered to pregnant mothers (fetuses), neonates, and infants.

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LETTER TO THE EDITOR

Canine hair as a model for tracing ethylmercury from Thimerosal-containing vaccines

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Key words: **dogs; ethylmercury; methylmercury; Thimerosal; vaccines; fish**

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Hair-Hg concentration (total or chemically speciated) is an accepted criterion for Hg monitoring related to exposure from fish and/or vaccines. Sedlackova *et al.* (2013) made a timely contribution modelling total Hg in hair of dogs to assess post-vaccine status with Thimerosal-containing vaccines (TCV), thus providing an opportunity to update relevant issues.

Recognizing the need to assay post-vaccine ethylmercury (EtHg), I support the rationale of Sedlackova *et al.* concurring that hair-Hg in dogs' hair should be carried out under assumptions valid for human hair. In this matter there are several aspects deserving attention:

- First, we should ask what the proportion of organic to inorganic Hg is in dogs' hair. It is assumed to be around 80% for methylmercury in humans. During the growing phase of hair (assuming that it is comparable to human), keratinocytes are the main tissue structure that capture Hg (Schoeman *et al.* 2010); under this assumption TCV-EtHg can end up in hair directly (see references 11 and 12 in Sedlackova *et al.*) or as inorganic Hg due to its instability, as suggested by Sedlackova *et al.* Nevertheless, we should also consider that not only do sulfur-keratin proteins in hair possess an affinity for Hg; other hair structures can also accumulate metals (Dórea & Pereira, 1983).
- In relation to post-vaccine change in hair-Hg concentrations, it was no surprise that they did not find significant differences. Because the total exposure of vaccine was not stated, and we were not informed the rate of hair growth and/or hair turnover, such crude measures allowed no adjustment amenable to kinetic interpretation. Indeed, in human hair, chemical speciation techniques have shown that the amount of EtHg captured after vaccination is minimal (see references 11 and 12 in Sedlackova *et al.*). Using this analogy, TCV-EtHg may not be quantitatively sufficient to appear in dogs' hair.
- Sedlackova *et al.* used a vaccine against rabies (with 0.01% of Thimerosal), but did not inform if the animals had been previously immunized with the other TCVs they mentioned (rabies; tetanus; leptospirosis; distemper; distemper and parvovirosis; infectious hepatitis; infectious laryngotracheitis; parvovirosis; and parainfluenza). Crucial for the kinetic of vaccine-EtHg, as recognized by the authors, is information on the dosage, i.e., that which we could have from weight of the animals (these data were not presented).
- There is another methodological issue worth reconsidering, i.e., statistical analysis. In the case of time-measure of the same variable a 'repeated measurements analysis' seems more appropriate to test retrospectively the sampling effect of time-related changes in total Hg concentrations. Additionally, because of intra and inter-variability of hair-Hg concentrations, I would also recommend integrating the data as function of time-0, i.e., recalculating all changes as a percentage of that initial value for all dogs individually.

- For the sake of contextualization, the median values of hair-Hg in Sedlackova *et al.* (23 to 52 ng/g) are in the lower range of those reviewed by Souza *et al.* (2013) in different parts of the world. Fish intake in sledge dog was associated with high values reported in most studies, thus indicating that methylmercury impacted hair-Hg concentrations. Additionally, while in humans the blood:hair ratio is around 250, in dogs these ratios have varied from 50 to 200 (references in Santos *et al.* 2013).
- Last, but not least, the authors discussed their data as a function of vaccine-Hg half-life in blood of human infants; they should consider that once in the human brain, inorganic-Hg half-life seems to be several years – five years or longer (Rooney 2013).

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Premature and neonate modeling of thimerosal exposure and neurodevelopment: additional comments

Health professionals in pediatrics, especially in neonatology, welcome the timely work of Chen et al.[1] Despite the widespread use of Thimerosal-containing vaccines (TCVs) in premature newborns, this is the first attempt to model exposure to thimerosal and neurodevelopment; that is why this study is both interesting and important. Given the special circumstances of the immature neurologic and immunologic systems of the premature-newborn, it is worth raising additional relevant issues to this revealing paper.

Metabolic stress is known to exacerbate Hg toxicity in rat model.[2] Body weight loss in human neonates (10%) occurs at the expense of body water and is more taxing in preterm babies;[2] neonates lose weight up to 5 days of life, but term-babies regain initial weight at a faster rate than preterm babies (10 days against 10 to 14 days respectively).

So far, all findings of low doses of ethylmercury relevant to vaccines demonstrated that infant animals (mice, rats, and monkeys) manifested neurological delays.[2] However, it is worth mentioning that another confounder not considered in any experimental study so far is TCV taken during pregnancy and other cumulative sources of environmental Hg exposure. Nowadays, expecting mothers are exposed to environmental Hg (fish-MeHg and dental amalgams) and likely to TCVs.

What is the vaccine equivalent of the modeled Thimerosal exposure? Actually Chen et al's Thimerosal doses used (32.2 μg/kg b.w. or 16 μgHg/kg b.w.) were close or relatively smaller than that actually reported (21.1 μgHg/kg b.w.) in premature babies;[3] additionally, the highest dose of Thimerosal (131.2 μg/kg b.w. or 65.6 μgHg/kg b.w.) was less than the cumulative doses received during the first six months, after correcting for weight gain.[3] In this regard, Chen et al's model is conservative and as a consequence the results are significant to model safety ranges of premature or neonate Hg exposure relevant to pediatric TCVs.

Before 2004, there are hardly any experimental studies addressing small doses of ethylmercury and neurological outcomes; these recent studies confirm well known impairment of nervous system functioning known to be caused by small doses of methylmercury.[2] It should be noticed that all these results focused on one mercury compound and a specific measured outcome. However, another important topic to consider in relation to experimental neurotoxicity of TCV is the obligatory occurrence of ethylmercury in association with aluminum as an adjuvant of such vaccines. In the specific case of hepatitis B vaccine, the ratio of Al:Hg is 20.[4] Given the susceptibility of young rats to aluminum toxicity[5] and translocation of intramuscular injection of alum-containing vaccine from muscle to the brain,[6] future experiments should explore the combined effect of Al plus Hg at ratios found in pediatric TCVs in order to address the actual range of combined Al and Hg in such vaccines used by the majority of infants in poor countries. Indeed, we are now learning that adjuvant-Al can produce neurological effects on its own,[7] but we have no clue as to the combined effect of ethylmercury plus adjuvant-Al as they occur in pediatric TCVs used in developing countries.

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We are grateful to your comments. The dose of thimerosal used in our study was according to the common mercury content in hepatitis B which was 12.5μg,[1] and converted into microgram per kilogram for rat by surface conversion formula. Next, according to the 1, 2, 3, 4 times, we designed 4 groups of thimerosal dose. Since thimerosal was dissolved in saline, we used saline group as a control in which main item was excluded for the saline interference.

We agreed that it was worth mentioning that aluminum is not considered in any thimerosal experimental study, which is an adjuvant of thimerosal-containing vaccines (TCVs). Given the reasons you suggested in the letter, we also strongly believed that it is urgent to study the combined effect of ethylmercury plus adjuvant-Al in TCVs. Of course, just as you said that TCV taken during pregnancy and other cumulative sources of environmental Hg exposure are not considered in experimental study now.

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Review

Exposure to Mercury and Aluminum in Early Life: Developmental Vulnerability as a Modifying Factor in Neurologic and Immunologic Effects

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Abstract: Currently, ethylmercury (EtHg) and adjuvant-Al are the dominating interventional exposures encountered by fetuses, newborns, and infants due to immunization with Thimerosal-containing vaccines (TCVs). Despite their long use as active agents of medicines and fungicides, the safety levels of these substances have never been determined, either for animals or for adult humans—much less for fetuses, newborns, infants, and children. I reviewed the literature for papers reporting on outcomes associated with (a) multiple exposures and metabolism of EtHg and Al during early life; (b) physiological and metabolic characteristics of newborns, neonates, and infants relevant to xenobiotic exposure and effects; (c) neurobehavioral, immunological, and inflammatory reactions to Thimerosal and Al-adjuvants resulting from TCV exposure in infancy. Immunological and neurobehavioral effects of Thimerosal-EtHg and Al-adjuvants are not extraordinary; rather, these effects are easily detected in high and low income countries, with co-exposure to methylmercury (MeHg) or other neurotoxicants. Rigorous and replicable studies (in different animal species) have shown evidence of EtHg and Al toxicities. More research attention has been given to EtHg and findings have showed a solid link with neurotoxic effects in humans; however, the potential synergic effect of both toxic agents has not been properly studied. Therefore, early life exposure to both EtHg and Al deserves due consideration.

Keywords: ethylmercury; methylmercury; aluminum; breastfeeding; Thimerosal; vaccines; neonates

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REVIEW PAPER

Abating Mercury Exposure in Young Children Should Include Thimerosal-Free Vaccines

José G. Dórea[1]

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Abstract Pediatric immunization is essential to prevent, control and eradicate children`s infectious diseases. Newborns and infants in less developed countries have a concentrated schedule of Thimerosal-containing vaccines (TCVs); pregnant mothers are also immunized with TCVs. Metabolic changes during early development are demonstrably an important risk factor for ethylmercury (EtHg) effects on neurodevelopment, while exposure to Thimerosal sensitizes susceptible individuals to life-long contact dermatitis. Concerns regarding toxicity of Hg have moved rich nations to withdraw it from medicines and, in particular, Thimerosal from pediatric vaccines; it has been more than 20 years since rich countries started using Thimerosal-free vaccines. TCVs and Thimerosal-free vaccines show dissimilar profiles of adverse effects. Thimerosal-free vaccines have shown a decrease in contact dermatitis, while TCVs showed a significant association with increased risk of tic disorders; in some circumstances, EtHg in combination with other neurotoxic substances negatively impacted neurobehavioral tests. In studies that explored vaccines and risk of tics, Thimerosal was a necessary factor. However, when the binary exposure to organic Hg forms (TCV–EtHg and fish-MeHg) was considered, effects on neurobehavioral tests were inconsistent. Conclusions: (a) The indiscriminate use of pediatric-TCVs in less developed countries carries an unjustifiable and excessive EtHg exposure with an unnecessary risk of neurotoxicity to the developing brain; (b) measurable benefits (of Thimerosal-free) and measurable risks of tic disorders have been associated with the (Thimerosal-containing) type of vaccine; (c) Thimerosal-free vaccines are clinically and toxicologically justifiable and they should be available to children in less developed countries.

Keywords Thimerosal-free vaccines · Ethylmercury · Infants · Contact dermatitis · Tic disorders

Introduction

The pathogenesis of Hg toxicity has received input from a wide range of in vitro and in vivo experimental studies. These have identified molecular and genetic factors driving the heterogeneity of immunological and neurobehavioral outcomes and the respective risks of exposure to all chemical-Hg forms [1]. Biochemical, clinical and epidemiologic studies indicate that small amounts of Thimerosal can lead to adverse effects [2]. Thimerosal, a preservative/adjuvant commonly used in vaccines is associated with an increased toxicological risk from pediatric Thimerosal-containing vaccines (TCVs). Therefore, concerns regarding the toxicity of Hg have found different solutions in regards to pediatric vaccines. Compared to the USA, which had Thimerosal in approximately 30 different childhood vaccines, France only had it in two (in 1999), and these two were also available in a Thimerosal-free formulation [3]. Nevertheless, the most developed nations have withdrawn Hg from medicines and, in particular Thimerosal from pediatric vaccines; it has been more than 20 years since developed countries started using Thimerosal-free vaccines [4].

Thimerosal has a limited role in immunogenicity (of intended antigens) but has a use in some vaccine manufacturing processes [4]. Thimerosal is a very active compound with a potential to act on the immunological and

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Low-dose Thimerosal in pediatric vaccines: Adverse effects in perspective

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ABSTRACT

Vaccines are prophylactics used as the first line of intervention to prevent, control and eradicate infectious diseases. Young children (before the age of six months) are the demographic group most exposed to recommended/mandatory vaccines preserved with Thimerosal and its metabolite ethylmercury (EtHg). Particularly in the less-developed countries, newborns, neonates, and young children are exposed to EtHg because it is still in several of their pediatric vaccines and mothers are often immunized with Thimerosal-containing vaccines (TCVs) during pregnancy. While the immunogenic component of the product has undergone more rigorous testing, Thimerosal, known to have neurotoxic effects even at low doses, has not been scrutinized for the limit of tolerance alone or in combination with adjuvant-Al during immaturity or developmental periods (pregnant women, newborns, infants, and young children). Scientific evidence has shown the potential hazards of Thimerosal in experiments that modeled vaccine-EtHg concentrations. Observational population studies have revealed uncertainties related to neurological effects. However, consistently, they showed a link of EtHg with risk of certain neurodevelopment disorders, such as tic disorder, while clearly revealing the benefits of removing Thimerosal from children's vaccines (associated with immunological reactions) in developed countries. So far, only rich countries have benefited from withdrawing the risk of exposing young children to EtHg. Regarding Thimerosal administered to the very young, we have sufficient studies that characterize a state of uncertainty: the collective evidence strongly suggests that Thimerosal exposure is associated with adverse neurodevelopmental outcomes. It is claimed that the continued use of Thimerosal in the less-developed countries is due to the cost to change to another preservative, such as 2-phenoxyethanol. However, the estimated cost increase per child in the first year of life is lower than estimated lifetime cost of caring for a child with a neurodevelopmental disorder, such tic disorder. The evidence indicates that Thimerosal-free vaccine options should be made available in developing countries.

1. Introduction

Vaccines are prophylactics used as the first line of intervention to prevent, control, and eradicate infectious diseases. Young children (before the age of 6 months) are the demographic group most exposed to recommended/mandatory vaccines that are preserved with Thimerosal and its metabolite ethylmercury (EtHg). Furthermore, in less-developed countries, this vulnerable demographic range (newborns, neonates, young children) is additionally exposed to EtHg when mothers are immunized with Thimerosal-containing vaccines (TCVs) during pregnancy (Dórea, 2011a). Indeed, in certain circumstances, six TCVs may be given to pregnant mothers: tetanus, up to three doses of hepatitis B, seasonal flu and H1N1 vaccines (Dórea, 2011a).

Modern vaccine development and evaluation require multiple stages involving academia, industry, and federal agencies (Curlin et al., 2011). Because not all immune components elicit an ideal response, vaccines are often formulated to contain an adjuvant, and when bottled in multi-dose vials, a preservative may be justified. In order to be manufactured, vaccines have to be formulated to resist contamination in the production line and during handling and application from multi-dose vials. As a result, some vaccines contain both preservative-Thimerosal and adjuvant-Al.

During vaccine production, no modern toxicity studies are required to detect specific aspects of low-dose EtHg (alone or in combination with Aluminum) in susceptible individuals; rather, non-specific toxicity tests such as body weight changes are frequently used (Sharma et al., 2012). Albeit at low doses, toxic ingredients (such as Thimerosal and adjuvant-Al) are intrinsically part of the vaccine's development and distribution but without modern toxi-testing assessment. Vaccines in general, be they TCVs or Thimerosal-free vaccines, are tested in adults for efficacy and safety related to their immunogenicity (Rebedea et al., 2006). Regardless of the formulation with and without Thimerosal, licensed vaccines show an extremely low rate of adverse events associated with the immune component (Ahmed et al., 2011). The

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Are toxic biometals destroying your children's future?

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Abstract Cadmium, arsenic, lead, and mercury have been linked to autism, attention deficit disorder, mental retardation and death of children. Mercury in thimerosal found in many vaccines and flu shots contributes significantly to these problems. Decomposition of the thimerosal can produce more toxic compounds, either methylethylmercury or diethylmercury, in the body. These compounds have a toxicity level similar to dimethylmercury. Within the human body, a mitochondrial disorder may release the more toxic form of mercury internally. Young children and pregnant women must minimize internal exposure to the vaccines and flu shots containing mercury.

Keywords Toxic metals · Thimerosal · Mercury · Premature birth · Autism

Introduction

From 1997 to 2002, the levels of mercury in the form of the preservative thimerosal found in childhood vaccines and flu shots were far too high based on levels of mercury permitted in edible food (fish, turtles, eels, etc.) by the EPA (Environmental Protection Agency) and FDA (Food and Drug Administration). Evidence of human bodies retaining specific mercury compounds causing different types of problems, and even death, has been known for a number of years. Mercury has been traditionally linked to autism, behavior disabilities and death (Autism News Staff 2008; Bennett and Autism Coach 2008).

The highest rates of autism and learning disabilities appeared in children born during the period of 1997–2003, and then the rate leveled out and has been reported to decrease during the past three years (Bennett and Autism Coach 2008; NewsMax.com Staff 2006). Another recent report indicates that the rate of autism has continued to increase at an average growth rate of 17% since 2003 (Thoughtful House 2009). Part of these increases can be attributed to improved identification of the problems and changes in the definition of autism.

Thimerosal, an organic complex containing mercury, was initially added to vaccines and flu shots and given to very young children in 1997. Since then, the annual rates of autism and learning disabilities appear to have increased more than 80 times the rates before the exposure of small children to thimerosal. A significant number of small children exhibited memory problems and an inability to function normally after they received a vaccine containing thimerosal.

During 2002–2004, levels of thimerosal (a preservative containing mercury bound to an ethyl group and a sulfur-benzoate type ring) were significantly reduced in required injections and medications for

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Behavioral and Brain Functions

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Review

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Mercury exposure, nutritional deficiencies and metabolic disruptions may affect learning in children

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Abstract

Among dietary factors, learning and behavior are influenced not only by nutrients, but also by exposure to toxic food contaminants such as mercury that can disrupt metabolic processes and alter neuronal plasticity. Neurons lacking in plasticity are a factor in neurodevelopmental disorders such as autism and mental retardation. Essential nutrients help maintain normal neuronal plasticity. Nutritional deficiencies, including deficiencies in the long chain polyunsaturated fatty acids eicosapentaenoic acid and docosahexaenoic acid, the amino acid methionine, and the trace minerals zinc and selenium, have been shown to influence neuronal function and produce defects in neuronal plasticity, as well as impact behavior in children with attention deficit hyperactivity disorder. Nutritional deficiencies and mercury exposure have been shown to alter neuronal function and increase oxidative stress among children with autism. These dietary factors may be directly related to the development of behavior disorders and learning disabilities. Mercury, either individually or in concert with other factors, may be harmful if ingested in above average amounts or by sensitive individuals. High fructose corn syrup has been shown to contain trace amounts of mercury as a result of some manufacturing processes, and its consumption can also lead to zinc loss. Consumption of certain artificial food color additives has also been shown to lead to zinc deficiency. Dietary zinc is essential for maintaining the metabolic processes required for mercury elimination. Since high fructose corn syrup and artificial food color additives are common ingredients in many foodstuffs, their consumption should be considered in those individuals with nutritional deficits such as zinc deficiency or who are allergic or sensitive to the effects of mercury or unable to effectively metabolize and eliminate it from the body.

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ORIGINAL PAPER

Administration of Thimerosal to Infant Rats Increases Overflow of Glutamate and Aspartate in the Prefrontal Cortex: Protective Role of Dehydroepiandrosterone Sulfate

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Abstract Thimerosal, a mercury-containing vaccine preservative, is a suspected factor in the etiology of neurodevelopmental disorders. We previously showed that its administration to infant rats causes behavioral, neurochemical and neuropathological abnormalities similar to those present in autism. Here we examined, using microdialysis, the effect of thimerosal on extracellular levels of neuroactive amino acids in the rat prefrontal cortex (PFC). Thimerosal administration (4 injections, i.m., 240 μg Hg/kg on postnatal days 7, 9, 11, 15) induced lasting changes in amino acid overflow: an increase of glutamate and aspartate accompanied by a decrease of glycine and alanine; measured 10–14 weeks after the injections. Four injections of thimerosal at a dose of 12.5 μg Hg/kg did not alter glutamate and aspartate concentrations at microdialysis time (but based on thimerosal pharmacokinetics, could have been effective soon after its injection). Application of thimerosal to the PFC in perfusion fluid evoked a rapid increase of glutamate overflow. Coadministration of the neurosteroid, dehydroepiandrosterone sulfate (DHEAS; 80 mg/kg; i.p.) prevented the thimerosal effect on glutamate and aspartate; the steroid alone had no influence on these amino acids. Coapplication of DHEAS with thimerosal in perfusion fluid also blocked the acute action of thimerosal on glutamate. In contrast, DHEAS alone reduced overflow of glycine and alanine, somewhat potentiating the thimerosal effect on these amino acids. Since excessive accumulation of extracellular glutamate is linked with excitotoxicity, our data imply that neonatal exposure to thimerosal-containing vaccines might induce excitotoxic brain injuries, leading to neurodevelopmental disorders. DHEAS may partially protect against mercurials-induced neurotoxicity.

Keywords Thimerosal · Glutamate · Amino acids · Microdialysis · DHEAS

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Introduction

Thimerosal, an organomercurial (THIM; sodium ethylmercurithiosalicylate), has been used as a preservative in liquid medicinal products, including pediatric vaccines, for decades without being adequately tested for safety in developing organisms. THIM, which contains approximately 49% mercury by weight and is composed of ethylmercury (EtHg) and thiosalicylic acid, is metabolized in the body to EtHg and further to inorganic forms of mercury [1]. Previous studies reported that THIM administration to rats leads to accumulation of mercury in the liver, kidneys and the brain, where it may produce toxic effects [2–6]. Although THIM was withdrawn from use in primary pediatric vaccines in most developed countries, it is still

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Genotoxicity of thimerosal in cultured human lymphocytes with and without metabolic activation sister chromatid exchange analysis proliferation index and mitotic index

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Abstract

Thimerosal is an antiseptic containing 49.5% of ethyl mercury that has been used for years as a preservative in many infant vaccines and in flu vaccines. Thimerosal is an organic mercurial compound used as a preservative in biomedical preparations. In this study, we evaluated the genotoxic effect of thimerosal in cultured human peripheral blood lymphocytes using sister chromatid exchange analysis in culture conditions with and without S9 metabolic activation. This study is the first report investigating the genotoxic effects of thimerosal in cultured human peripheral blood lymphocyte cells using sister chromatid exchange analysis. An analysis of variance test (ANOVA) was performed to evaluate the results. Significant induction of sister chromatid exchanges was seen at concentrations between 0.2 and 0.6 μg/ml of thimerosal compared with negative control. A significant decrease ($p < 0.001$) in mitotic index (MI) and proliferation ındex (PRI) as well as an increase in SCE frequency ($p < 0.001$) was observed compared with control cultures. Our results indicate the genotoxic and cytotoxic effect of TH in cultured human peripheral blood lymphocytes at tested doses in cultures with/without S9 fraction.

Keywords: Genotoxicity; Thimerosal; S9 activation system; Mitotic index; Proliferation index

1. Introduction

Thimerosal (TH, sodium ethylmercury-thiosalicylate,) was developed by Eli Lilly in the 1930 s as an effective bacteriostatic, fungistatic preservative and has been widely used in multidose vials of vaccines and in ophthalmic, otic, nasal, and topical products. (Ball et al., 2001). TH contains 49.6% mercury by weight and releases ethylmercury as a metabolite. In the body, ethylmercury can be converted to inorganic mercury. Inorganic mercury is known to induce membrane and DNA damage (Ferrat et al., 2002; Ben-Ozer et al., 2000), and in cell culture conditions it was shown to be mutagenic and generate DNA breaks. Due to possible adverse health effects, investigations on its metabolism and toxicity are urgently needed. An *in vivo* study on chronic toxicity of TH in rats was inconclusive and reports on genotoxic effects in various *in vitro* and/or *in vivo* systems were contradictory. Little is known about the reactions of human peripheral blood lymphocyte at low concentrations, which can occur after using TH containing products.

In addition, there were reports on genotoxic effects of TH *in vivo*. A weak but significant increase in micronuclei and chromosome aberrations was seen in male Swiss CD–1 mice at doses between 10 and 20 mg/kg (Marrazzini et al., 1994); another study in used male and female (102/E1·C3H/E1) F1 mice and Swiss albino mice reported negative results (Adler et al., 1991).

Possible carcinogenic effects were investigated in one study on the chronic toxicity of TH in Fischer 344 rats (Mason et al., 1971). However, this study does not meet

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Mercury toxicokinetics—dependency on strain and gender

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ABSTRACT

Mercury (Hg) exposure from dental amalgam fillings and thimerosal in vaccines is not a major health hazard, but adverse health effects cannot be ruled out in a small and more susceptible part of the exposed population. Individual differences in toxicokinetics may explain susceptibility to mercury. Inbred, H-2-congenic A.SW and B10.S mice and their F1- and F2-hybrids were given $HgCl_2$ with 2.0 mg Hg/L drinking water and traces of ^{203}Hg. Whole-body retention (WBR) was monitored until steady state after 5 weeks, when the organ Hg content was assessed. Despite similar Hg intake, A.SW males attained a 20–30% significantly higher WBR and 2- to 5-fold higher total renal Hg retention/concentration than A.SW females and B10.S mice. A selective renal Hg accumulation but of lower magnitude was seen also in B10.S males compared with females. Differences in WBR and organ Hg accumulation are therefore regulated by non-H-2 genes and gender. Lymph nodes lacked the strain- and gender-dependent Hg accumulation profile of kidney, liver and spleen. After 15 days without Hg A.SW mice showed a 4-fold higher WBR and liver Hg concentration, but 11-fold higher renal Hg concentration, showing the key role for the kidneys in explaining the slower Hg elimination in A.SW mice. The trait causing higher mercury accumulation was not dominantly inherited in the F1 hybrids. F2 mice showed a large inter-individual variation in Hg accumulation, showing that multiple genetic factors influence the Hg toxicokinetics in the mouse. The genetically heterogeneous human population may therefore show a large variation in mercury toxicokinetics.

Introduction

All living organisms are exposed to low levels of mercury due to its eternal presence in the environment, but exposure leading to health hazards are generally related to specific human activities and of multitude origin: from unintentional occupational exposure (Rohling and Demakis, 2006), to ingestion of Hg as an ingredient in folk remedies, religious attributes, and skin-lightening creams (Pollard and Hultman, 2007; Risher and De Rosa, 2007). However, the main forms of Hg exposure recently discussed (Clarkson and Magos, 2006) as a source of adverse health effects are amalgam fillings (inorganic Hg) (Bates, 2006; Martin and Woods, 2006), food, especially fish (methyl Hg) (Grandjean et al., 2003; Passos et al., 2007), and as a preservative in vaccines (thimerosal) (Clifton, 2007).

A number of recent studies in cohorts of humans exposed in the above ways (Bellinger et al., 2006; de Burbure et al., 2006; DeRouen et al., 2006; Passos et al., 2007; Woods et al., 2007, 2008; Barregard et al., 2008; Pichichero et al., 2008) have tried to establish association between exposure to mercury and any adverse health effects. In addition, case reports of disease conditions after various forms of Hg exposure regularly appear in the medical literature (Mahaffey, 2005; Risher and De Rosa, 2007). Furthermore, experiments in mammals (Havarinasab et al., 2007) also including non-human primates (Burbacher et al., 2005) have been used to increase the knowledge of Hg toxicokinetics and related health effects.

The majority of the studies on these forms of Hg exposure have concluded that there is no clear evidence for significant health effects except in special situations such as methyl mercury exposure due to high consumption of Hg-rich fish and seafood (Grandjean et al., 2003). However, increased urinary Hg excretion (Woods et al., 2007; Dunn et al., 2008; Ye et al., 2008) , and increased urinary protein excretion (microalbuminiuria) has been described following exposure from dental amalgam fillings (Barregard et al., 2008). However, even after studies in large exposed cohorts, uncertainty remains with regard to possible adverse health effects due to Hg exposure in susceptible individuals (Barregard, 2005; Bellinger et al., 2008). Individual susceptibility may be due to unexpected high exposure caused for example by gum chewing or bruxism in dental amalgam bearers (Sallsten et al., 1996; Isacsson et al., 1997; Barregard et al., 2008), or genetic factors causing differences in the toxicokinetics of

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The Effect of Thimerosal on Neutrophil Migration

A Comparison with the Effect on Calcium Mobilization and CD11b Expression

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ABSTRACT. The sulfhydryl-reactive compound thimerosal caused a chemotactic stimulation of neutrophil migration at low concentrations and inhibition of chemoattractant-stimulated chemotaxis at high concentrations. Thiosalicylic acid, an analog of thimerosal devoid of mercury, also stimulated migration at low concentrations and caused inhibition at higher concentrations, though the inhibitory effect was less pronounced than that of thimerosal. These results indicate that the stimulatory effect of thimerosal on migration is due to the thiosalicylic acid moiety of the molecule. In contrast with thimerosal which, especially at higher concentrations than required for optimal stimulation of migration, caused an increase in cytosolic free calcium ($[Ca^{2+}]_i$), thiosalicylic acid had no effect on $[Ca^{2+}]_i$ of the neutrophil. This suggests that the presence of mercury is decisive for the calcium-mobilizing effect, but not for stimulation of migration, and that mobilization of calcium and activation of migration are not related. Thimerosal caused a strong increase of CD11b expression in neutrophils in suspension, especially at inhibitory concentrations, while thiosalicylic acid had no effect on CD11b expression. This could mean (but does not prove) that CD11b expression is more related to the calcium-mobilizing effect of thimerosal than to its stimulation of migration. BIOCHEM PHARMACOL **55**;3: 305–312, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. neutrophil; thimerosal; migration; chemotaxis; calcium; CD11b expression

Migration by neutrophils plays a predominant role in both the anti-microbial and the inflammation-promoting activities of these cells by enabling them to reach the site of infection or inflammation. In spite of extensive research, the molecular basis of the migration process remains largely unknown, with the calcium homeostasis during migration being a matter of particular controversy [1–4]. Most chemoattractants cause an increase in cytosolic free calcium ($[Ca^{2+}]_i$)†, but there is no evidence that the ability to cause an increase in $[Ca^{2+}]_i$ is related to the extent of migration. On the contrary, chemotactic migration is inhibited by a number of agents which cause an increase in $[Ca^{2+}]_i$ [5–9].

Thimerosal is an organomercury compound with sulfhydryl-reactive properties. It is clinically used as a topical antiinfective agent because of its antibacterial and antifungal properties. The substance has a profound effect on calcium homeostasis in a number of cells. While it was originally described as an agent having a specific effect on inositol trisphosphate (IP_3)-sensitive calcium stores, recent studies have shown that in addition to IP_3-sensitive stores, ryanodine-sensitive stores are also affected by thimerosal [10, 11]. The effect of thimerosal is biphasic: at low concentrations it causes cytosolic calcium oscillations in endothelial cells, whereas at high concentrations the oscillations are inhibited and a sustained increase in $[Ca^{2+}]_i$ is observed [12].

Thimerosal causes an increase of $[Ca^{2+}]_i$ in neutrophils [13]. It also causes a strong increase in 5-lipoxygenase metabolites when another activator, such as formyl-methionyl-leucyl-phenylalanine (fMLP), is present [13, 14]. Leukotriene formation depended on the presence of extracellular $[Ca^{2+}]_i$, and it was concluded that the enhancing effect of thimerosal on fMLP-induced leukotriene formation was due to its modulating effect on calcium homeostasis.

Because of the effects of thimerosal on calcium homeostasis and because of sulfur-containing compounds were shown in previous studies to be capable of inducing chemotaxis, we decided to study the effect of thimerosal on neutrophil migration. We set out to determine whether the substance could induce chemotaxis by itself and whether thimerosal could affect chemotactic migration activated by other chemoattractants. In addition, we wished to address the question as to whether the effect on migration was connected with the effect on calcium metabolism. To determine the importance of mercury in the thimerosal molecule, we compared the results of thimerosal with those of thiosalicylic acid, a mercury-less analog of thimerosal (Fig. 1).

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† *Abbreviations:* fMLP, formyl-methionyl-leucyl-phenylalanine; $[Ca^{2+}]_i$, cytosolic free calcium concentration; IL-8, interleukin 8; IP_3, inositol trisphosphate; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; LTB_4: leukotriene B_4.

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Review article

Thimerosal
A versatile sulfhydryl reagent, calcium mobilizer, and cell function-modulating agent

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Abstract

An overview of the literature concerning the effects of thimerosal is presented. Because of its antibacterial effect, thimerosal is used for a variety of practical purposes such as antiseptic and preservative. In biomedical studies, thimerosal is used as a sulfhydryl reagent, and as a calcium-mobilizing agent. The ability of thimerosal to act as a sulfhydryl group is related to the presence of mercury. Relatively little study has been devoted to the mechanism of the reaction of thimerosal with the sulfhydryl group; the sulfhydryl reactive capacity is mostly concluded on the basis of inactivation of the effect by dithiothreitol (DTT). Thimerosal causes a release of calcium from intracellular stores in many cells types; this is followed by an influx of extracellular calcium. Both InsP3- and ryanodine-sensitive calcium stores may be affected. Studies with permeabilized cells or organelles show that the effect of thimerosal on calcium is dependent on the concentration: low concentrations of thimerosal stimulate calcium release, high concentrations are inhibitory. This dependence is not found in intact cells. Thimerosal may activate or inhibit a number of cell functions. These are often related to the ability to release calcium or with the sulfhydryl reactivity. In platelets, thimerosal causes aggregation, increase of arachidonic acid metabolism, and exocytotic release of serotonin. In neutrophils, thimerosal causes, besides an increase of cytosolic free calcium, an increase of formyl-methionyl-leucyl-phenylalanine (fMLP)-activated leukotriene release, and a modulation of chemotactic migration and exocytosis. At low concentrations, thimerosal induces chemotactic migration of neutrophils, in the absence of other chemoattractants. The effect is also observed with thiosalicylic acid, indicating that the stimulation of migration was due to the thiosalicylic acid moiety of the thimerosal molecule. At higher concentrations, thimerosal causes inhibition of fMLP-activated migration. Low concentrations of thimerosal, but not of thiosalicylic acid, induced exocytotic enzyme release from neutrophils. High concentrations of thimerosal inhibited fMLP-activated exocytosis. The results point to an involvement of calcium mobilization and calcium influx of activation, and reaction with sulfhydryl groups for inhibition.

Keywords: Thimerosal; Calcium; Sulfhydryl group; Neutrophil; Chemotaxis; Exocytosis

Thimerosal—also named thiomersal, merthiolate or sodium ethylmercuri-thiosalicylate—is a water-soluble derivative of thiosalicylic acid, with antibacterial and antifungal properties. The two important parts of the molecule are the thiosalicylic acid moiety, and the possession of mercury coupled to sulfur (Fig. 1). These two parts determine most of the properties of thimerosal. The carboxylic group of thiosalicylic acid makes the compound water-soluble because at physiological pH the compound is present as a sodium salt. Thimerosal is able to penetrate the cell membrane; it enters the cell as the free acid which is formed in the equilibrium with the charged carboxylate form. As a consequence the rate of penetration of thimerosal is pH-dependent. The mercury atom gives the compound oxidative character. The relative importance of the mercury and the thiosalicylic acid part can be easily determined by comparing the effects of thimerosal with those of thiosalicylic acid.

Thimerosal has two main applications. One group of applications is of a practical nature, and is related with the antibacterial and antifungal effect of thimerosal. It is widely used as an antiseptic agent and as a preservative in topical medicaments, cleaning solutions for eye lenses, cosmetics, and vaccines. The other application is in the biomedical laboratory. In the latter case the chemical properties of thimerosal, notably its ability to react as a sulfhydryl reagent, are used to study basic mechanisms in cell physiology, especially calcium homeostasis and processes related to the effect on calcium.

The practical applications of thimerosal are accom-

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Comparative Toxicity of Preservatives on Immortalized Corneal and Conjunctival Epithelial Cells

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Abstract

Purpose: Nearly all eye drops contain preservatives to decrease contamination. Nonpreservatives such as disodium-ethylene diamine tetra-acetate (EDTA) and phosphate-buffered saline are also regularly added as buffering agents. These components can add to the toxicity of eye drops and cause ocular surface disease. To evaluate the potential toxicity of these common components and their comparative effects on the ocular surface, a tissue culture model utilizing immortalized corneal and conjunctival epithelial cells was utilized.

Methods: Immortalized human conjunctival and corneal epithelial cells were grown. At confluency, medium was replaced with 100 µL of varying concentrations of preservatives: benzalkonium chloride (BAK), methyl paraben (MP), sodium perborate (SP), chlorobutanol (Cbl), and stabilized thimerosal (Thi); varying concentrations of buffer: EDTA; media (viable control); and formalin (dead control). After 1 h, solutions were replaced with 150 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazonium bromide). After 4 h, solutions decanted, 100 µL of acid isopropanol added, and the optical density determined at 572 nm to evaluate cell viability.

Results: Conjunctival and corneal cell toxicity was seen with all preservatives. Depending upon concentration, BAK exhibited from 56% to 89% toxicity. In comparison, Cbl exhibited from 50% to 86%, MP from 30% to 76%, SP from 23% to 59%, and Thi from 70% to 95%. EDTA with minimal toxicity (from 6% to 59%) was indistinguishable from SP.

Conclusions: Generally, the order of decreasing toxicity at the most commonly used concentrations: Thi (0.0025%) > BAK (0.025%) > Cbl (0.25%) > MP (0.01%) > SP (0.0025%) ≈ EDTA (0.01%). Even at low concentration, these agents will cause some degree of ocular tissue damage.

Introduction

Most eye drops contain preservatives to provide a level of antimicrobial activity in the bottle, limiting secondary bacterial, mycotic, and amoebal ocular infections caused by contaminated solutions and prolong the half-life of the drug by preventing biodegradation and maintaining drug potency.[1] Preservatives can be classified into four main categories: detergents, oxidants, chelating agents, and metabolic inhibitors (pentavalent antimonials [Sb^V], quaternary ammoniums, and organomercurials).[2,3] Examples of such preservatives include: benzalkonium chloride (BAK; detergent), chlorobutanol (Cbl; detergent), methyl paraben (MP; chelating agent), sodium perborate (SP; oxidative agent), and stabilized thimerosal (Thi; organomercurial); although by far, the most common of the topical ophthalmic medication preservatives is BAK, typically used in concentrations varying from 0.015% to 0.05%. Disodium-ethylene diamine tetra-acetate (EDTA) and phosphate-buffered saline, while not preservatives, are added to most ophthalmic formulations as buffering agents. While stabilizing agents such as buffers are generally thought of as nontoxic, the potential for toxicity still exists. In fact any chemical added to eye drops, such as the preservative and buffering agents just mentioned, have the potential to harm the eye.[1] Toxicity from pharmaceutical agents can result in decreased visual acuity and/or patient comfort that can lead to decreased compliance.

Benzalkonium chloride (BAK) stabilizes drugs in solution and prevents spoilage by microbial growth; but it can also initiate ocular surface damage and subconjunctival inflammation.[1,4] BAK is a detergent preservative that can affect cell membrane permeability, interrupt the metabolic processes of the cell, cause lysis of cell contents, and allow

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REGULATION OF SODIUM CURRENTS THROUGH OXIDATION AND REDUCTION OF THIOL RESIDUES

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Abstract—Changes in redox state are involved in several physiological and pathophysiological processes. Previous experiments have demonstrated that nitric oxide can function as a reactive oxygen species, inhibiting neuronal sodium currents by nitrosylation of thiol residues. We hypothesized that nitric oxide and thiol oxidizers similarly modulate voltage-dependent sodium currents. Voltage-dependent sodium currents were studied with the whole-cell patch-clamp technique in NB41A3 neuroblastoma cells. The nitric oxide donor 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine did not affect sodium currents. In contrast, the thiol oxidizers thimerosal and 4,4′-dithiopyridine significantly inhibited sodium currents. The effect of thimerosal persisted after washout, but could be fully reversed by the reducing agent dithiothreitol. Reduced glutathione did not restore the sodium current amplitude when given extracellularly, while intracellular glutathione prevented the inhibitory effect of thimerosal. Pretreatment with the alkylating agent *N*-ethylmaleimide blocked the inhibitory action of thimerosal. Thiol oxidation caused a shift in the voltage dependence of fast and slow inactivation to more hyperpolarized potentials without concomitant effects on the voltage dependence of activation. Mercaptoethanol and reduced glutathione enhanced sodium currents by shifting the voltage dependence of inactivation to depolarized potentials.

These results demonstrate that the oxidation and reduction of thiol residues alters the properties of voltage-sensitive sodium channels and may play an important role in the regulation of membrane excitability. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: neuroblastoma cells, thiol oxidation, slow inactivation.

Post-translational modification of ion channels plays an important role in fine-tuning cellular function of excitable and non-excitable cells. Phosphorylation of serine or threonine residues, as well as tyrosine phosphorylation, have been studied extensively.[5] The covalent modification of ion channels through protein kinases and phosphatases significantly alters their properties, thereby changing membrane function and excitability. More recently, several studies have demonstrated that changes in redox state may contribute to the control of cell function.[4,19,20] Oxidation of cysteine or methionine residues by reactive oxygen species affects ligand-gated and voltage-dependent ion channels in a variety of different cells.[1,8,10,11,18,25,26] Such reactive oxygen species are generated during inflammatory processes and reperfusion injury, and contribute to the structural and functional damage seen in these disorders.[2,6,7] In addition, changes in cellular redox state have been observed during signaling processes, suggesting a potential role of reactive oxygen species in second messenger cascades.[14,22]

We have recently demonstrated that nitric oxide (NO) inhibits voltage-dependent sodium currents in vagal sensory neurons.[3,19] NO caused this inhibition by functioning as a reactive oxygen species, because this effect was due to nitrosylation of cysteine residues and did not depend on the activation of guanylate cyclase. As nodose neurons express NO synthase, NO may function as an autocrine modulator of neuronal activity. Vagal sensory neurons from the nodose ganglion express at least two different sodium channels that differ in their biophysical and pharmacological properties. Both were equally affected by NO.[19] To identify whether NO alters sodium currents in another cell, we investigated the effects of NO in NB41A3 cells, a murine neuroblastoma cell line. Moreover, we hypothesized that oxidation of thiol groups similarly inhibits sodium currents. Previous studies have demonstrated that NB41A3 cells express tyrosine hydroxylase, a marker for cells of neuronal lineage.[13] Even in the absence of specific neurotrophic factors, rapidly activating voltage-dependent sodium currents can be recorded from cells that are round in appearance and do not have long processes.[27] Therefore, this murine cell line appeared to be an approprite model system for the study of ion channel modulation in neuronal cells.

EXPERIMENTAL PROCEDURES

Cell culture

We used the neuroblastoma cell line NB41A3 (CCl-147; American Type Culture Collection, Manasses, VA) for all experiments. The cells were cultured in Ham's F10 supplemented with 2 mM L-glutamine, 1.5 g/l $NaHCO_3$ and 10% horse serum. Cells were passaged in poly-L-lysine-coated flasks after five to seven days. Two days before performing the electrophysiological experiments, the cells were plated on to poly-L-lysine-coated coverslips. At the time of the experiment, most cells were round or triangular, with no or few short processes, minimizing space clamp problems that might interfere with the voltage control of the cells.

Electrophysiological recordings

The cells attached to the coverslips were transferred into a 0.5-ml recording chamber on the stage of an inverted microscope (Nikon). Sodium currents were recorded using the whole-cell patch-clamp technique with an Axopatch 200A amplifier (Axon Instruments, CA)

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Abbreviations: DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether)tetra-acetate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; NO, nitric oxide; papaNONOate, 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine; TTX, tetrodotoxin.

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Organ mercury levels in infants with omphaloceles treated with organic mercurial antiseptic

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SUMMARY Samples of fresh and fixed tissues from infants with exomphalos treated by thiomersal application were analysed for mercury content. The results showed that thiomersal can induce blood and organ levels of organic mercury which are well in excess of the minimum toxic level in adults and fetuses. The analysis of fresh and fixed tissues must be carefully controlled against normal tissues in order to interpret mercury levels accurately.

The introduction of the application of 0·1% tincture of Thimerosal (thiomersal)* in the treatment of exomphalos is generally attributed to Grob (1957). No unequivocal cases of organic mercury poisoning have been reported after its use in patients with exomphalos though several cases of 'pink disease' have been reported (Schippan and Wehran, 1968; Stanley-Brown and Frank, 1971), as well as a case of 'mercury intoxication' (Leenders *et al.*, 1974). This is thought to be an idiosyncratic reaction unrelated to excessive dosage and should be distinguished from true poisoning.

Analysis of fresh tissue samples obtained at necropsy from an infant with exomphalos treated by thiomersal application who died unexpectedly showed raised tissue levels of mercury. This prompted us to search the records at the Hospital for Sick Children, Toronto, for other mercury-treated cases of exomphalos and carry out organ mercury analysis.

Materials and methods

Between 1969 and 1975 there had been 13 cases of exomphalos treated by thiomersal application. 10 had died and 9 of these had necropsy examinations. Formalin-fixed wet tissues were available from 6 of the 9. Mercury assays had been carried out in 1972 on fresh tissues from 2 of these 6 cases by the Public Health Division of the Department of Health, Toronto, using similar methods to those described below.

We performed organ mercury assays on three sets of fresh tissue samples and six sets of formalin-fixed tissues. Cold vapour atomic absorption was used to measure total mercury in blood (Magos and Clarkson, 1972). Solid tissues were weighed and homogenized in 0·9% w/v sodium chloride solutions (1·0 g tissue to 9 ml sodium chloride solution). Aliquots from the homogenate were treated and measured as described for hair samples by Giovanoli-Jakubczak *et al.* (1974). Samples of the mercury-contaminated omphalocele sac were *not* stored or transported in the same container as any of the analysed samples.

Results

Table 1 shows that all 3 cases in which fresh tissue analysis was performed had absorbed an excessive load of mercury ranging from 65 to 2700 times the normal tissue levels. The fresh organ levels in Cases 2 and 3 suggest that the blood levels were similar to or, perhaps in Case 3, even higher than the level of 1340 ppb (parts per billion) found in Case 1. Mercury assays were repeated on the formalin-fixed tissues of the 3 cases in which fresh tissue assays had been performed, and the results are shown in Table 2 with those from the other fixed and stored samples.

These results show a general increase in mercury concentration after fixation which appears to be related more to the duration of storage than to the total dose administered. The mercury content of the formalin fixative was negligible, <6 ppb. Although analysis of all the samples indicated an excessive load

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*The sodium salt of orthocarboxyphenylthioethyl mercury, or sodium mercurithiosalicylate, is the active ingredient of Merthiolate, Lilly, and contains approximately 49% mercury by weight.

ETHYLMERCURITHIOSALICYLATE – A NEW REAGENT FOR THE STUDY OF PHOSPHATE TRANSPORT IN MITOCHONDRIA

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1. Introduction

The physiological function of mitochondria requires the transport of P_i across the inner mitochondrial membrane for 3 main metabolic reactions: (1) Uptake of phosphate together with ADP for the synthesis of ATP within the matrix; (2) Exchange of phosphate against dicarboxylates for metabolite fluxes between cytosol and matrix; (3) Uptake and release of phosphate together with Ca^{2+} for calcium homeostasis in the cytoplasm [1]. For these functions 4 separate phosphate transport systems have been described in mitochondria which could only be differentiated by their sensitivity against SH-group inhibitors: (1) The electroneutral phosphate/proton symporter is inhibited by MalNEt and low concentrations of *p*-chloromercuribenzoate and mersalyl [2–4]; (2) The electroneutral dicarboxylate antiporter, exchanging dicarboxylates or phosphate against each other, is inhibited by *p*-chloromercuribenzoate, mersalyl and the dicarboxylate analogon butylmalonate, but not by MalNEt [5–7]; (3) The electrogenic phosphate uniporter is inhibited by mersalyl [8] and with inverted inner membrane vesicles by *p*-chloromercuribenzoate and MalNEt [9]; (4) The electrogenic calcium/phosphate symporter was found insensitive against MalNEt and mersaylyl [10,11]. The latter transport system, however, does not seem to represent a separate transporter, because the insensitivity against MalNEt and *p*-chloromercuribenzoate could not be corroborated [12,13].

The simultaneous occurrence of the electroneutral phosphate/proton symporter (phosphate uptake) and the electrogenic phosphate uniporter (phosphate release) in mitochondria, would result in a futile cycle, driven by the mitochondrial proton pump. Therefore a strong regulation of the phosphate transport system has to be assumed. The existence of only one regulated transport system which functions either as proton/phosphate symporter or as phosphate/dicarboxylate antiporter has also been suggested [14–16]. Here the effect of the antiseptic ethylmercurithiosalicylate (thiomersal), which contains sulfur and mercury in a covalent linkage, on the phosphate uptake of mitochondria is described. The data suggest a regulated sensitivity of the phosphate/proton symport against SH-inhibitors.

Abbreviations: PMS, *p*-chloromercuri phenylsulfonate; MalNEt, *N*-ethylmaleimide; SDS, sodium dodecylsulfate

2. Materials and methods

MalNEt, thiomersal and thiosalicylic acid were purchased from Serva (Heidelberg), PMS and rotenone from Sigma (St Louis). [^{32}P]Phosphate and [^{3}H]-sucrose (3 Ci/mmol) were obtained from Amersham Buchler. All other chemicals were of analytical grade.

Rat liver mitochondria were isolated by standard procedures [17]. Swelling of mitochondria was done as in [16], either in 100 mM ammonium phosphate (pH 7.3), 2 mM EDTA and 1 μM rotenone (A), or in 80 mM potassium phosphate (pH 7.3), 40 mM ammonium chloride, 1 μM rotenone (B). The uptake of [^{32}P]phosphate was measured in aliquots (800 μl) taken from cuvettes containing 2 ml swelling medium (B) and 0.2 μCi [^{32}P]phosphate plus 2 μCi [^{3}H]-sucrose, at the indicated times after addition of mitochondria. After centrifugation for 30 s in an Eppendorf centrifuge the supernatant was immediately removed and the pellet dissolved in 300 μl 2% SDS and counted in 10 ml scintillation fluid. Protein was determined by the biuret method [18].

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MINI REVIEW
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Chemicals, Nutrition, and Autism Spectrum Disorder: A Mini-Review

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The rapid increase of the prevalence of autism spectrum disorder (ASD) suggests that exposure to chemicals may impact the development of ASD. Therefore, we reviewed literature on the following chemicals, nutrient to investigate their association with ASD: (1) smoke/tobacco, (2) alcohol, (3) air pollution, (4) pesticides, (5) endocrine-disrupting chemicals, (6) heavy metals, (7) micronutrients, (8) fatty acid, and (9) parental obesity as a proxy of accumulation of specific chemicals or nutritional status. Several chemical exposures such as air pollution (e.g., particular matter 2.5), pesticides, bisphenol A, phthalates, mercury, and nutrition deficiency such as folic acid, vitamin D, or fatty acid may possibly be associated with an increased risk of ASD, whereas other traditional risk factors such as smoking/tobacco, alcohol, or polychlorinated biphenyls are less likely to be associated with ASD. Further research is needed to accumulate evidence on the association between chemical exposure and nutrient deficiencies and ASD in various doses and populations.

Keywords: autism spectrum disorder, air pollution, chemicals, pesticide, fatty acid, micronutrients, heavy metal, environment

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INTRODUCTION

Autism spectrum disorder (ASD) is a developmental disorder typified by impaired communication and social skills (Grabrucker, 2012). A recent increase in cases of ASD from 4–5 of 10,000 persons in 1966 to 100 cases of 10,000 persons currently (Fombonne, 2009) may not solely be explained by genetic factors (Abrahams and Geschwind, 2010). Thus, it needs to be determined whether environmental factors play a role in the onset of ASD (Grabrucker, 2012), and a recent study using twin samples reported that around 50% of cases of ASD can be explained by environmental factors (Hallmayer et al., 2011).

In the present mini-review, we report several relatively new studies that have evaluated the association between ASD and environmental factors by focusing on chemical or nutritional exposures because these are modifiable factors. These exposures included smoking/tobacco, alcohol, air pollution, pesticides, endocrine-disrupting chemicals, heavy metals, micronutrients, and fatty acid. Parental obesity was also included as an exposure because maternal obesity can be an indicator of exposure to chemicals or nutrition.

SMOKE OR TOBACCO

Although not consistent, most recent population-based studies have suggested that maternal smoking during pregnancy is not directly associated with ASD after adjusting for socioeconomic status (Burstyn et al., 2010; Kalkbrenner et al., 2012; Lee et al., 2012; Tran et al., 2013). For example,

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Review Article

Neuropathology and Animal Models of Autism: Genetic and Environmental Factors

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Autism is a heterogeneous behaviorally defined neurodevelopmental disorder. It is defined by the presence of marked social deficits, specific language abnormalities, and stereotyped repetitive patterns of behavior. Because of the variability in the behavioral phenotype of the disorder among patients, the term autism spectrum disorder has been established. In the first part of this review, we provide an overview of neuropathological findings from studies of autism postmortem brains and identify the cerebellum as one of the key brain regions that can play a role in the autism phenotype. We review research findings that indicate possible links between the environment and autism including the role of mercury and immune-related factors. Because both genes and environment can alter the structure of the developing brain in different ways, it is not surprising that there is heterogeneity in the behavioral and neuropathological phenotypes of autism spectrum disorders. Finally, we describe animal models of autism that occur following insertion of different autism-related genes and exposure to environmental factors, highlighting those models which exhibit both autism-like behavior and neuropathology.

1. Introduction

Autism is a heterogeneous neurodevelopmental disorder with multiple causes and a great range in the severity of symptoms [1, 2]. As described by Kanner in 1943, individuals with autism have four core features: (i) impairments in reciprocal social interactions, (ii) an abnormal development in the use of language, (iii) repetitive and ritualized behaviors, and (iv) a narrow range of interests [3]. These symptoms range from mild to severe as defined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition (DSM-IV) [4] (Figure 1). In addition to the core features, people with autism often have comorbid neurological disorders such as mental retardation and epilepsy [5]. The prevalence of mental retardation with autism is ~60%, but in the broader autism spectrum disorders (ASDs), the number is closer to 30% [6]. Epilepsy has been long associated with autism although estimates of the occurrence of seizure disorder vary from 5% to 44% [7]. Anxiety and mood disorders are very common in autism [8]. There is also a substantial heterogeneity in the onset of autism. Impairments in some children manifest before 18 months of age; however, 25%–40% of children with autism initially demonstrate near normal development until 18–24 months, when they regress into autism that is generally indistinguishable from the early onset form of the disorder [8]. The early onset versus regressive phenotypes of autism suggest different neuropathological mechanisms.

Neuropathological observations that have emerged over the past decade point towards early pre- and postnatal developmental abnormalities that involve multiple regions of the brain, including the cerebellum, cortical white matter, amygdala, brain stem, and cerebral cortex. However, since 1980, only 120 postmortem brains from people with autism have been studied [9]. Thus, the neuropathology literature is neither extensive nor rigorous, and there are several areas that remain open to further investigation. In the present review, we have highlighted neuropathological features of the areas that may play an important role in the pathology of autism.

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HEPATITIS B VACCINATION OF MALE NEONATES AND AUTISM DIAGNOSIS, NHIS 1997–2002

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Universal hepatitis B vaccination was recommended for U.S. newborns in 1991; however, safety findings are mixed. The association between hepatitis B vaccination of male neonates and parental report of autism diagnosis was determined. This cross-sectional study used weighted probability samples obtained from National Health Interview Survey 1997–2002 data sets. Vaccination status was determined from the vaccination record. Logistic regression was used to estimate the odds for autism diagnosis associated with neonatal hepatitis B vaccination among boys age 3–17 years, born before 1999, adjusted for race, maternal education, and two-parent household. Boys vaccinated as neonates had threefold greater odds for autism diagnosis compared to boys never vaccinated or vaccinated after the first month of life. Non-Hispanic white boys were 64% less likely to have autism diagnosis relative to nonwhite boys. Findings suggest that U.S. male neonates vaccinated with the hepatitis B vaccine prior to 1999 (from vaccination record) had a threefold higher risk for parental report of autism diagnosis compared to boys not vaccinated as neonates during that same time period. Nonwhite boys bore a greater risk.

Universal newborn immunization with the hepatitis B vaccination was recommended in 1991 (CDC, 1991). A recent narrative review concluded that hepatitis B vaccines available since 1982 are safe and effective (Demirjian & Levy, 2009); however, safety findings from individual studies are mixed. In Vaccine Safety Datalink studies, Lewis et al. (2001) reported no evidence of a significant association between vaccination at birth and fever or neurological adverse events, Naleway et al. (2009) found an elevated, although not statistically significant, risk of immune hemolytic anemia in children vaccinated with hepatitis B vaccine, and Price et al. (2010) reported no association between autism and vaccination with the hepatitis B vaccination during the first month of life. Additionally, Marques et al. (2007) found no association between time of hepatitis B vaccination, i.e., within 24 hrs versus 2–4 days postnatally, and neurodevelopment delays at 6 months of age. In contrast, increased risk for central nervous system inflammatory demyelination in childhood were associated with hepatitis B vaccination (Mikaeloff et al., 2009). Further, hepatitis B vaccination has been associated with acute ear infection and pharyngitis, chronic arthritis (Fisher et al., 2001), and liver problems, such as jaundice (Fisher & Eklund, 1999), as well as

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In this unfunded study, any analyses, interpretations, or conclusions reached are those of the authors, not the National Center for Health Statistics, which is responsible only for data collection.
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Differential immunotoxic effects of inorganic and organic mercury species *in vitro*

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Abstract

Despite the fact that humans are exposed to multiple forms of mercury (elemental, inorganic, and organic), most research on mercury toxicity has focused on methylmercury (MeHg) and on neurotoxic outcomes and mechanisms. Recent work has indicated that the immunotoxic effects of mercury compounds may be significant contributors to human disease as well as mechanistically relevant to other target organ toxicities. In this study, we compared the effects of inorganic Hg (iHg) to organic Hg species (MeHg and ethylmercury, EtHg) in human peripheral blood mononuclear cells (PBMCs) *in vitro* at sub -cytotoxic concentrations, using methods developed to characterize response of human PBMCs to iHg *in vitro*. PBMCs were isolated from six volunteer blood donors (3 males, 3 females) and cultured in the presence and absence of lipopolysaccharide (LPS) and low levels (up to 200 nM of each Hg species, separately) for 24 hours in culture. Cell culture supernatants were analyzed for cytokine concentrations with a bead-based multiplex assay.

We report that iHg and MeHg both increase pro-inflammatory cytokine release in LPS-stimulated PBMCs, while EtHg decreases IFN-γ release as well pro-inflammatory cytokine release. IL-17 release is significantly increased only in response to iHg treatment. Levels of anti-inflammatory cytokines (IL-1Ra and IL-10) were not significantly altered by any Hg treatment. These results indicate that both organic and inorganic species of Hg can affect the human immune system, but that they may exert different effects on immune function.

Keywords

mercury; immunotoxicity; humans; *in vitro*; cytokines

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The authors declare that there are not conflicts of interest.

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The plausibility of a role for mercury in the etiology of autism: a cellular perspective

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Autism is defined by a behavioral set of stereotypic and repetitious behavioral patterns in combination with social and communication deficits. There is emerging evidence supporting the hypothesis that autism may result from a combination of genetic susceptibility and exposure to environmental toxins at critical moments in development. Mercury (Hg) is recognized as a ubiquitous environmental neurotoxin and there is mounting evidence linking it to neurodevelopmental disorders, including autism. Of course, the evidence is not derived from experimental trials with humans but rather from methods focusing on biomarkers of Hg damage, measurements of Hg exposure, epidemiological data, and animal studies. For ethical reasons, controlled Hg exposure in humans will never be conducted. Therefore, to properly evaluate the Hg-autism etiological hypothesis, it is essential to first establish the biological plausibility of the hypothesis. This review examines the plausibility of Hg as the primary etiological agent driving the cellular mechanisms by which Hg-induced neurotoxicity may result in the physiological attributes of autism. Key areas of focus include: (1) route and cellular mechanisms of Hg exposure in autism; (2) current research and examples of possible genetic variables that are linked to both Hg sensitivity and autism; (3) the role Hg may play as an environmental toxin fueling the oxidative stress found in autism; (4) role of mitochondrial dysfunction; and (5) possible role of Hg in abnormal neuroexcitory and excitotoxity that may play a role in the immune dysregulation found in autism. Future research directions that would assist in addressing the gaps in our knowledge are proposed.

Keywords: autism; mercury; cellular; oxidative stress; mitochondrial; immune dysfunction

Introduction

Rather than critically examining the extensive literature relating to the possible role of Hg in the etiology of autism, this review focused specifically on the biological plausibility of the hypothesis from a cellular perspective. This is an essential prerequisite for furthering our understanding of Hg's possible role in autism because, for ethical reasons, one cannot conduct experimental Hg trials on humans. Instead, one needs to rely on the broad discipline of epidemiology to bring together disparate lines of inquiry and methodologies that inform the area. Independent and *a priori* of this, it is of fundamental importance to evaluate the biological plausibility of the hypothesis. A body of epidemiological evidence

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A comparative evaluation of the effects of MMR immunization and mercury doses from thimerosal--containing childhood vaccines on the population prevalence of autism

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Potential conflict of interest: Dr. Mark Geier has been an expert witness and a consultant in cases involving adverse reactions to vaccines before the U.S. Vaccine Compensation Act and in civil litigation. David Geier has been a consultant in cases involving adverse reactions to vaccines before the U.S. Vaccine Compensation Act and in civil litigation.

Summary

Background: The purpose of the study was to evaluate the effects of MMR immunization and mercury from thimerosal-containing childhood vaccines on the prevalence of autism.

Material/Methods: Evaluations of the Biological Surveillance Summaries of the Centers for Disease Control and Prevention (CDC), the U.S. Department of Education datasets, and the CDC's yearly live birth estimates were undertaken.

Results: It was determined that there was a close correlation between mercury doses from thimerosal--containing childhood vaccines and the prevalence of autism from the late 1980s through the mid-1990s. In contrast, there was a potential correlation between the number of primary pediatric measles-containing vaccines administered and the prevalence of autism during the 1980s. In addition, it was found that there were statistically significant odds ratios for the development of autism following increasing doses of mercury from thimerosal-containing vaccines (birth cohorts: 1985 and 1990–1995) in comparison to a baseline measurement (birth cohort: 1984). The contribution of thimerosal from childhood vaccines (>50% effect) was greater than MMR vaccine on the prevalence of autism observed in this study.

Conclusions: The results of this study agree with a number of previously published studies. These studies have shown that there is biological plausibility and epidemiological evidence showing a direct relationship between increasing doses of mercury from thimerosal-containing vaccines and neurodevelopmental disorders, and measles-containing vaccines and serious neurological disorders. It is recommended that thimerosal be removed from all vaccines, and additional research be undertaken to produce a MMR vaccine with an improved safety profile.

key words: autism • ethylmercury • MMR • neurodevelopmental disorders • thimerosal

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An assessment of the impact of thimerosal on childhood neurodevelopmental disorders

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Keywords Autism, mental retardation, speech disorders, VAERS.

Summary

The prevalence of autism in the US has risen from 1 in ~2500 in the mid-1980s to 1 in ~300 children in the mid-1990s. The purpose of this study was to evaluate whether mercury from thimerosal in childhood vaccines contributed to neurodevelopmental disorders. Neurodevelopmental disorder dose-response curves for increasing mercury doses of thimerosal in childhood vaccines were determined based upon examination of the Vaccine Adverse Events Reporting System (VAERS) database and the 2001 US' Department of Education Report. The instantaneous dosage of mercury children received in comparison to the Food and Drug Administration (FDA)'s maximum permissible dose for the oral ingestion of methylmercury was also determined. The dose-response curves showed increases in odds ratios of neurodevelopmental disorders from both the VAERS and US Department of Education data closely linearly correlated with increasing doses of mercury from thimerosal-containing childhood vaccines and that for overall odds ratios statistical significance was achieved. Similar slopes and linear regression coefficients for autism odds ratios in VAERS and the US Department of Education data help to mutually validate each other. Controls employed in the VAERS and US Department of Education data showed minimal biases. The evidence presented here shows that the occurrence of neurodevelopmental disorders following thimerosal-containing childhood vaccines does not appear to be coincidental.

Introduction

Thimerosal is an organic mercury compound. It is metabolized to ethylmercury and thiosalicylate and has been present since the 1930s as a preservative in many vaccines and pharmaceutical products to prevent bacterial and fungal contamination.

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One has published the first epidemiological evidence showing a direct association between thimerosal-containing childhood vaccines and neurodevelopmental disorders in children [1, 2]. It has been shown that there was from a 2–6-fold statistically significant increased incidence of neurodevelopment disorders following an additional 75–100 microgram dosage of mercury from thimerosal-containing childhood vaccines in comparison to thimerosal-free childhood vaccines [1]. One has also shown that there were dose-response curves demonstrating a close correlation between increasing mercury doses from childhood vaccines and childhood neurodevelopmental disorders [2].

The purpose of this study was to extend previous studies and integrate statistical and dose-response curve methodologies into a single analysis evaluating mercury doses from childhood vaccines and childhood neurodevelopmental disorders. In the first part of this study, the dose-response was evaluated of increasing mercury doses from thimerosal-containing Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccine in comparison to thimerosal-free DTaP vaccines for neurodevelopmental disorders from 1997–2001, based upon examination of the Vaccine Adverse Events Reporting System (VAERS) database. Secondly, the 2001 US' Department of Education Report [3] was evaluated on the prevalence of neurodevelopmental disorders and the average dosage of mercury that children received as part of their childhood immunization schedules in birth cohorts in comparison to a baseline measurement. The final part of this analysis studied the instantaneous dosage of mercury children received in comparison to the Food and Drug Administration (FDA)'s maximum permissible dose for the oral ingestion of methylmercury as part of the 2002 recommended childhood immunization schedule. It was determined by the FDA in 1999 that, under the recommended childhood immunization schedule, infants might be exposed to cumulative doses of ethylmercury that exceed some federal safety guidelines established for oral ingestion of methylmercury [4].

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Neurodevelopmental Disorders Following Thimerosal-Containing Childhood Immunizations: A Follow-Up Analysis

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The authors previously published the first epidemiological study from the United States associating thimerosal from childhood vaccines with neurodevelopmental disorders (NDs) based upon assessment of the Vaccine Adverse Event Reporting System (VAERS). A number of years have gone by since their previous analysis of the VAERS. The present study was undertaken to determine whether the previously observed effect between thimerosal-containing childhood vaccines and NDs are still apparent in the VAERS as children have had a chance to further mature and potentially be diagnosed with additional NDs. In the present study, a cohort of children receiving thimerosal-containing diphtheria-tetanus-acellular pertussis (DTaP) vaccines in comparison to a cohort of children receiving thimerosal-free DTaP vaccines administered from 1997 through 2000 based upon an assessment of adverse events reported to the VAERS were evaluated. It was determined that there were significantly increased odds ratios (ORs) for autism (OR = 1.8, $p < .05$), mental retardation (OR = 2.6, $p < .002$), speech disorder (OR = 2.1, $p < .02$), personality disorders (OR = 2.6, $p < .01$), and thinking abnormality (OR = 8.2, $p < .01$) adverse events reported to the VAERS following thimerosal-containing DTaP vaccines in comparison to thimerosal-free DTaP vaccines. Potential confounders and reporting biases were found to be minimal in this assessment of the VAERS. It was observed, even though the media has reported a potential association between autism and thimerosal exposure, that the other NDs analyzed in this assessment of the VAERS had significantly higher ORs than autism following thimerosal-containing DTaP vaccines in comparison to thimerosal-free DTaP vaccines. The present study provides additional epidemiological evidence supporting previous epidemiological, clinical and experimental evidence that administration of thimerosal-containing vaccines in the United States resulted in a significant number of children developing NDs.

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Potential conflict of interest: David Geier has been a consultant in cases involving vaccines before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. Dr. Mark Geier has been a consultant and an expert witness in cases involving vaccines before the no-fault NVICP and in civil litigation.

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Keywords Autistic Spectrum Disorders, Ethylmercury, Merthiolate, Thiomersal, VAERS

We previously published the first epidemiological evidence from the United States showing an association between thimerosal-containing childhood vaccines and neurodevelopmental disorders (Geier and Geier 2003a). Specifically, it was determined that there was from a two- to sixfold statistically significantly increased reporting rate of neurodevelopmental disorders, depending on the specific symptom or disorder, to the Vaccine Adverse Event Reporting System (VAERS) database following thimerosal-containing diphtheria-tetanus-acellular pertussis (DTaP) (administered from 1992 to 2000) in comparison to thimerosal-free DTaP vaccine (administered from 1997 to 2000), whereas control adverse events were reported similarly following both vaccines under study.

In light of the fact that a number of years have gone by, the present study was undertaken to determine whether the previously observed effect between thimerosal-containing childhood vaccines and neurodevelopmental disorders are still apparent in the VAERS database as children have had a chance to further mature and potentially be diagnosed with a neurodevelopmental disorder. The results of the present analysis should allow one to be able to determine whether the previous observations represented a transient artifact, or whether the previous results are indeed robust, representing a true effect of thimerosal-containing childhood vaccines on neurodevelopmental disorders.

MATERIALS AND METHODS

The VAERS database is an epidemiological database that has been maintained by the Centers for Disease Control and Prevention (CDC) since 1990. Specific vaccine-adverse events following vaccination are required to be reported to this database as mandated by law. The VAERS Working Group of the CDC has previously reported that less than 5% of the total adverse events submitted to VAERS are reported by parents. The VAERS Working Group of the CDC and the Food and Drug Administration (FDA) analyze and publish epidemiologic studies based

Early Downward Trends in Neurodevelopmental Disorders Following Removal of Thimerosal-Containing Vaccines

David A. Geier, B.A.
Mark R. Geier, M.D., Ph.D.

ABSTRACT

Contemporaneously with the epidemic rise in neurodevelopmental disorders (NDs), first observed in the United States during the 1990s, the childhood immunization schedule was expanded by the U.S. Centers for Disease Control and Prevention (CDC) to include several additional thimerosal-containing vaccines (TCVs). On July 7, 1999, a joint recommendation was made by the American Academy of Pediatrics (AAP) and the U.S. Public Health Service (PHS) to remove thimerosal from vaccines. A two-phase study was undertaken to evaluate trends in diagnosis of new NDs entered into the Vaccine Adverse Event Reporting System (VAERS) and the California Department of Developmental Services (CDDS) databases on a reporting quarter basis, from 1994 through 2005. Significant increasing trends in newly diagnosed NDs were observed in both databases 1994 through mid-2002. Significant decreasing trends in newly diagnosed NDs were observed in both databases from mid-2002 through 2005. The results indicate that the trends in newly diagnosed NDs correspond directly to the expansion and subsequent contraction of the cumulative mercury dose to which children were exposed from TCVs through the U.S. immunization schedule.

Background

In 2004, the Department of Health and Human Services and the American Academy of Pediatrics (AAP) issued an Autism A.L.A.R.M., stating that 1 in 166 children currently have an autistic disorder, and 1 in 6 children have a developmental and/or behavioral disorder. Autism, once rare, is now more prevalent than childhood cancer, diabetes, and Down syndrome.[1] Epidemic trends in neurodevelopmental disorders (NDs) were first observed in the United States during the 1990s,[1-8] and cannot be explained by immigration, changed diagnostic criteria, or improved identification.[1,6-8]

Autism is an ND characterized by impairments in social relatedness and communication, repetitive behaviors, and stereotypic abnormal movements.[1] While genetic factors are important in the pathogenesis of autistic disorders, a role for environmental factors has received considerable attention.

Exposure to mercury has previously been shown to cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with autistic disorders, and with similarities in neuroanatomy, neurotransmitters, and biochemistry.[9-12] Furthermore, recent research that codes children's communicative, social, affective, repetitive behaviors, and toy play from videotapes of the toddlers' first and second birthday parties demonstrates that the regression associated with autistic disorders clearly manifests between the ages of 12 and 24 months,[13] concurrent with the exposure to thimerosal-containing childhood vaccines (TCVs).

Thimerosal is an ethylmercury-containing compound (49.6% mercury by weight) that was historically added to many vaccines at the preservative level (0.005% to 0.01%). The U.S. Centers for Disease Control and Prevention (CDC), from the late 1980s through the 1990s, expanded the number of doses of TCVs to be administered to U.S. infants. To five doses of diptheria-tetanus-whole-cell-pertussis (DTP) vaccine were added three doses of hepatitis B (Hep b) vaccine and four of *Haemophilus influenzae* type b (Hib) vaccine. Additionally, the CDC began recommending three doses of influenza vaccine for certain infant populations. An infant who received all of these vaccines on schedule could have received as much as 200 micrograms (µg) of mercury during the first 6 months of life.[14]

In response to theoretical concerns about the cumulative doses of mercury from TCVs, the AAP and the U.S. Public Health Service (PHS) issued a joint statement on July 7, 1999, calling for the removal of thimerosal from all vaccines.[14] It has been estimated that the last thimerosal-containing Hep b, diphtheria-tetanus-acellular-pertussis (DTaP) and Hib vaccines were manufactured in 2000-2001 and expired at the end of 2002 (or early 2003).[14] Table 1 summarizes significant historical dates in the use of pediatric TCVs in the United States.

Considering all significant environmental exposures to mercury, such as through breast milk, TCVs represent almost 50% of the total mercury dose some infants received.[15] The 187.5 µg of mercury through TCVs plus the average of 164 µg from breast milk during the first 6 months exceeded the methylmercury safety guidelines established by the U.S. Environmental Protection Agency (EPA), Health Canada, the World Health Organization (WHO), the Agency for Toxic Substances Disease Registry (ATSDR), and the U.S. Food and Drug Administration (FDA).[15] With no additional exposure from any source, these doses also exceeded the methylmercury guidelines for the first year of life set by all of these agencies except the FDA.[15]

Despite its removal from many childhood vaccines, thimerosal is still routinely added to some formulations of influenza vaccine administered to U.S. infants, as well as to several other vaccines (e.g. tetanus-diphtheria and monovalent tetanus) administered to older children and adults. In 2004, the Institute of Medicine (IOM) of the U.S. National Academy of Sciences (NAS) retreated from the stated 1999 goal of the AAP and the PHS to remove thimerosal from U.S. vaccines as soon as possible.[16] Furthermore, many nations still add thimerosal to many of their pediatric vaccines, and WHO and several vaccine manufacturers still advocate the continued use of thimerosal in pediatric vaccines. As a result, assessing the safety of TCVs is a matter of significant importance.

Examinations of the Vaccine Adverse Event Reporting System (VAERS), the U.S. Department of Education, and the Vaccine Safety Datalink (VSD) databases showed significant links between exposure to TCVs and NDs.[17,2-3] Specifically, data from VAERS showed that additional doses of mercury from thimerosal-containing DTaP in comparison to thimerosal-free DTaP (administered in the late 1990s), and additional doses of thimerosal-containing DTP and Hib in comparison to *diphtheria-*

A meta-analysis epidemiological assessment of neurodevelopmental disorders following vaccines administered from 1994 through 2000 in the United States

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Key words: **developmental delay; mercury; merthiolate; thimerosal; thiomersal**

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Abstract

BACKGROUND: Thimerosal is an ethylmercury-containing compound (49.6% mercury by weight) used as at the preservative level in vaccines (0.005% to 0.01%).

METHODS: Statistical modeling in a meta-analysis epidemiological assessment of the Vaccine Adverse Event Reporting System (VAERS) for neurodevelopment disorders (NDs) reported following Diphtheria-Tetanus-whole-cell-Pertussis (DTP) vaccines in comparison to Diphtheria-Tetanus-whole-cell-Pertussis-Haemophilus Influenzae Type b (DTPH) vaccines (administered: 1994–1997) and following Thimerosal-containing Diphtheria-Tetanus-acellular-Pertussis (DTaP), vaccines in comparison to Thimerosal-free DTaP vaccines (administered: 1997–2000), was undertaken.

RESULTS: Significantly increased adjusted (sex, age, vaccine type, vaccine manufacturer) risks of autism, speech disorders, mental retardation, personality disorders, thinking abnormalities, ataxia, and NDs in general, with minimal systematic error or confounding, were associated with TCV exposure.

CONCLUSION: It is clear from the results of the present epidemiological study and other recently published data associating mercury exposure with childhood NDs, additional ND research should be undertaken in the context of evaluating mercury-associated exposures, especially from Thimerosal-containing vaccines.

Conflict of interests: Dr. Mark Geier has been an expert witness and consultant in vaccine cases before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. David Geier has been a consultant in vaccine cases before the no-fault NVICP and in civil litigation.
Study Funding: None.

REVIEW ARTICLE

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A Prospective Assessment of Porphyrins in Autistic Disorders: A Potential Marker for Heavy Metal Exposure

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Autism was recently associated with a urinary porphyrin pattern indicative of mercury toxicity in a large cohort of French children. The IRB of the Institute for Chronic Illnesses approved the present study. A total of 37 consecutive American patients (≥7 years-old) with autism spectrum disorders (ASDs) (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition-DSM IV), born from 1983-1998, that presented to the Genetic Centers of America for outpatient genetic evaluations were prospectively examined for urinary prophryin levels (LabCorp, Inc.) from June 2005-June 2006. Imaging and laboratory testing were conducted on each patient to rule-out other causal factors for their ASDs. As controls, age-, sex-, and race-matched neurotypical ASD siblings were examined. An apparent dose-response effect was observed between autism severity and increased urinary coproporphyrins. Patients with non-chelated autism (2.25-fold, 83% had levels > 2 SD above the control mean) and non-chelated ASDs (2-fold, 58% had levels > 2 SD above the control mean), but not patients with non-chelated pervasive developmental delay-not otherwise specified (PDD-NOS) or Asperger's disorder (1.4-fold, 46% had levels > 2 SD above the control mean), had significantly increased median coproporphyrin levels versus controls. A significant increase (1.7-fold) in median coproporphyrin levels was observed among non-chelated ASD patients versus chelated ASD patients. Porphyrins should be routinely clinically measured in ASDs, and potential ASD treatments should consider monitoring porphyrin levels. Additional research should be conducted to evaluate the potential role for mercury exposure in some ASDs.

Keywords: Autistic; Chelation; Developmental Delay

INTRODUCTION

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairments in social relatedness and communication, repetitive behaviors, abnormal movement patterns, and sensory dysfunction (Eigsti and Shapiro, 2003; Werner and Dawson, 2005). While genetic factors are recognized as being important in the pathogenesis of ASDs, a role for environmental factors has received considerable attention. Several recent epidemiological studies have associated mercury exposure with ASDs (Counter *et al.*, 2002; Holmes *et al.*, 2003; Geier and Geier, 2005; Palmer *et al.*, 2006; Windham *et al.*, in press), and it has been reported that exposure to mercury can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with ASDs, and that these similarities extend to neuroanatomy, neurotransmitters, and biochemistry (Faustman *et al.*, 2000; Bernard *et al.*, 2001; 2002; Redwood et al, 2001; Blaxill *et al.*, 2004). Furthermore, a recent review has suggested that ASD children have been found to have significantly higher exposure to mercury than controls, and ASD children have been determined to have significantly increased body-burdens of mercury resulting from biochemical and genomic susceptibilities within detoxification pathways (Mutter *et al.*, 2005).

Recently, Nataf *et al.* (2006) have examined urinary porphyrin levels in a large series of children with autistic disorders from France. These researchers observed a porphyrin pattern among children with ASDs that implicated environmental toxicity, especially mercury toxicity, in childhood autistic disorders. The purpose of the present study was to conduct an examination of urinary porphyrin levels in a series of American patients with ASDs, so as to evaluate the consistency of the observations made by Nataf *et al.* in France with clinical observations made in the United States.

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A prospective study of thimerosal-containing Rho(D)-immune globulin administration as a risk factor for autistic disorders

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Abstract

Background. This study evaluated the relationship between prenatal mercury exposure from thimerosal (49.55% mercury by weight)-containing Rho(D)-immune globulins (TCRs) and autism spectrum disorders (ASDs).

Methods. The Institutional Review Board of the Institute for Chronic Illnesses approved the present study. A total of 53 consecutive non-Jewish Caucasian patients with ASDs (*Diagnostic and statistical manual of mental disorders, fourth ed.* – DSM IV) born between 1987 and 2001 who presented to the Genetic Centers of America for outpatient genetic/developmental evaluations were prospectively collected from June 1, 2005 through March 31, 2006. Imaging and laboratory testing were conducted on each patient to rule out other causal factors for their ASDs. As race-matched controls, the frequency of Rh negativity was determined from 926 non-Jewish Caucasian pregnant women who had presented for outpatient prenatal genetics care to the Genetic Centers of America between 1980 and 1989.

Results. Children with ASDs (28.30%) were significantly more likely (odds ratio 2.35, 95% confidence interval 1.17–4.52, $p < 0.01$) to have Rh-negative mothers than controls (14.36%). Each ASD patient's mother was determined to have been administered a TCR during her pregnancy.

Conclusion. The results provide insights into the potential role prenatal mercury exposure may play in some children with ASDs.

Keywords: *Developmental delay, ethylmercury, rhogam, thimerosal, thiomersal*

Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairments in social relatedness and communication, repetitive behaviors, abnormal movement patterns, and sensory dysfunction [1,2]. While genetic factors are recognized as being important in the pathogenesis of ASDs, a role for environmental factors has received considerable attention. For example, Beversdorf et al. reported that pathological changes in the cerebellum in autism are thought to correspond to an event before 30–32 weeks of gestation [3]. These researchers determined that a higher incidence of prenatal stressors was found in autism at 21–32 weeks of gestation, with a peak at 25–28 weeks, and concluded that their data supported the possibility of prenatal stressors as a potential contributor to autism. Additionally, researchers reported that exposure to mercury can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with autistic disorders, and that these similarities extend to neuroanatomy, neurotransmitters, and biochemistry [4–7].

Rho(D)-immune globulin is an immune globulin preparation containing antibodies to Rho(D) that is intended for intramuscular injection. Prior to late 2002/early 2003 when the last doses of thimerosal-containing Rho(D)-immune globulin preparations expired, most formulations of Rho(D)-immune globulin contained thimerosal. Thimerosal is an ethylmercury-containing compound (49.55% mercury by weight) that was added to Rho(D)-immune globulin preparations at the preservative level of 0.003–0.01%. Rho(D)-immune globulin is used to prevent isoimmunization in the Rho(D)-negative individual exposed to Rho(D) positive blood as a result of fetomaternal hemorrhage occurring during delivery of an Rho(D) positive infant, abortion (either spontaneous or induced), or following amniocentesis or abdominal trauma. Rh hemolytic

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An assessment of downward trends in neurodevelopmental disorders in the United States following removal of thimerosal from childhood vaccines

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Potential conflict of interest: David Geier has been a consultant in vaccine cases before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. Dr. Mark Geier has been an expert witness and a consultant in vaccine cases before the no-fault NVICP and in civil litigation

Summary

Background: The US is in the midst of an epidemic of neurodevelopmental disorders (NDs). Thimerosal is an ethylmercury-containing compound added to some childhood vaccines. Several previous epidemiological studies conducted in the US have associated Thimerosal-containing vaccine (TCV) administration with NDs.

Material/Methods: An ecological study was undertaken to evaluate NDs reported to the Vaccine Adverse Event Reporting System (VAERS) from 1991 through 2004 by date of receipt and by date of vaccine administration. The NDs examined included autism, mental retardation, and speech disorders. Statistical trend analysis was employed to evaluate the effects of removal of Thimerosal on the proportion of NDs reported to VAERS.

Results: There was a peak in the proportion of ND reports received by VAERS in 2001–2002 and in the proportion of ND reports by date of vaccine administration in 1998. There were significant reductions in the proportion of NDs reported to VAERS as Thimerosal was begun to be removed from childhood vaccines in the US from mid-1999 onwards.

Conclusions: The present study provides the first epidemiological evidence showing that as Thimerosal was removed from childhood vaccines, the number of NDs has decreased in the US. The analysis techniques utilized attempted to minimize chance or bias/confounding. Additional research should be conducted to further evaluate the relationship between TCVs and NDs. This is especially true because the handling of vaccine safety data from the National Immunization Program of the CDC has been called into question by the Institute of Medicine of the National Academy of Sciences in 2005.

key words: **Autism Spectrum Disorder • Merthiolate • Thimerasol • Thiomersal**

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AN EVALUATION OF THE EFFECTS OF THIMEROSAL ON NEURODEVELOPMENTAL DISORDERS REPORTED FOLLOWING DTP AND Hib VACCINES IN COMPARISON TO DTPH VACCINE IN THE UNITED STATES

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Thimerosal is an ethylmercury (49.55% mercury by weight) preservative historically added to some vaccines. Toxicokinetic studies showed children in the United States received doses of mercury from Thimerosal-containing vaccines (TCVs) in excess of safety guidelines. In the United States during the 1990s, diphtheria–tetanus–pertussis (DTP) and Haemophilus influenzae *type b (Hib) vaccines (maximally, 50 μg mercury per joint administration) and diphtheria–tetanus–pertussis–*Haemophilus influenzae *type b (DTPH) vaccines (25 μg mercury per administration) were given to children in the same childhood vaccination schedule at 2, 4, 6, and 15–18 mo, so that children receiving DTP and Hib vaccines may have maximally received an additional 100 μg more mercury exposure from TCVs than children administered DTPH vaccines. A case-control epidemiological study of neurodevelopmental disorders (NDs) reported to the Vaccine Adverse Event Reporting System (VAERS) (online public access version; updated 31 August 2004) following administration of DTP vaccines in comparison DTPH vaccines manufactured by Lederle Laboratories (Pearl River, NY) from 1994 through 1998 was undertaken. Significantly increased odds ratios for autism, speech disorders, mental retardation, infantile spasms, and thinking abnormalities reported to VAERS were found following DTP vaccines in comparison to DTPH vaccines with minimal bias or systematic error. Additional ND research should be undertaken in the context of evaluating mercury-associated exposures, especially since in 2005 the Institute of Medicine issued a report calling into question handling of vaccine safety data by the National Immunization Program of the Centers for Disease Control and Prevention.*

Thimerosal, an ethylmercurial preservative (49.55% mercury by weight) historically added to many vaccines, may have represented a significant source of mercury exposure in susceptible children (Ball et al., 2001). Thimerosal is still routinely added to required vaccines administered to U.S. infants (e.g., for influenza), and the Institute of Medicine (2004) of the U.S. National Academy

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Dr. Mark Geier has been an expert witness and consultant in cases involving vaccines before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. David Geier has been a consultant in cases involving vaccines before the no-fault NVICP and in civil litigation.
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A Case Series of Children with Apparent Mercury Toxic Encephalopathies Manifesting with Clinical Symptoms of Regressive Autistic Disorders

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Impairments in social relatedness and communication, repetitive behaviors, and stereotypic abnormal movement patterns characterize autism spectrum disorders (ASDs). It is clear that while genetic factors are important to the pathogenesis of ASDs, mercury exposure can induce immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with ASDs. The Institutional Review Board of the Institute for Chronic Illnesses (Office for Human Research Protections, U.S. Department of Health and Human Services, IRB number IRB00005375) approved the present study. A case series of nine patients who presented to the Genetic Centers of America for a genetic/developmental evaluation are discussed. Eight of nine patients (one patient was found to have an ASD due to Rett's syndrome) (a) had regressive ASDs; (b) had elevated levels of androgens; (c) excreted significant amounts of mercury post chelation challenge; (d) had biochemical evidence of decreased function in their glutathione pathways; (e) had no known significant mercury exposure except from Thimerosal-containing vaccines/Rho(D)-immune globulin preparations; and (f) had alternate causes for their regressive ASDs ruled out. There was a significant dose-response relationship between the severity of the regressive ASDs observed and the total mercury dose children received from Thimerosal-containing vaccines/Rho (D)-immune globulin preparations. Based upon differential diagnoses, 8 of 9 patients examined were exposed to significant mercury from Thimerosal-containing biologic/vaccine preparations during their fetal/infant developmental periods, and subsequently, between 12 and 24 mo of age, these previously normally developing children suffered mercury toxic encephalopathies that manifested with clinical symptoms consistent with regressive ASDs. Evidence for mercury intoxication should be considered in the differential diagnosis as contributing to some regressive ASDs.

Autism is a neurodevelopmental syndrome characterized by impairments in social relatedness and communication, repetitive behaviors, and stereotypic abnormal movement patterns (California Department of Developmental Services, 2003). While genetic factors are recognized as being important in the pathogenesis of autistic disorders, the role for environmental factors has received considerable attention. Researchers have previously reported that exposure to mercury can produce immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with autistic disorders, and these similarities extend to neuroanatomy, neurotransmitters, and biochemistry (Bernard et al., 2001, 2002; Blaxill et al., 2004; Redwood et al., 2001). Furthermore, recent research observing children's communicative, social, affective and repetitive behaviors and toy play coded from videotapes of the toddlers' first and second birthday parties revealed there are children with regressive autistic disorders that manifest between the ages of 12 and 24 mo (Werner & Dawson, 2005), a temporal period concurrent with exposure of these children to mercury from Thimerosal-containing biologics/vaccines in the U.S. standard immunization schedule.

MATERIALS AND METHODS

Participants

The Institutional Review Board of the Institute for Chronic Illnesses (Office for Human Research Protections, U.S. Department of Health and Human Services, IRB number IRB00005375) approved the present study. This study examines the cases of nine pediatric patients with neurodevelopmental disorders who presented to the Genetic Centers of America from June 2005 through February 2006 for outpatient genetic/developmental evaluations.

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David Geier has been a consultant in vaccine/biologic cases before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. Dr. Mark Geier has been an expert witness and a consultant in vaccine/biologic cases before the no-fault NVICP and in civil litigation.

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A Prospective Study of Mercury Toxicity Biomarkers in Autistic Spectrum Disorders

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Porphyrins are derivatives formed in the heme synthesis pathway and porphyrins afford a measure of xenobiotic exposure. The steps in the heme pathway most vulnerable to heavy metal inhibition are uroporphyrin decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX) reactions. Mercury toxicity was associated with elevations in urinary coproporphyrin (cP), pentacarboxyporphyrin (5cxP), and precoproporphyrin (prcP) (also known as keto-isocoproporphyrin) levels. Two cohorts of autistic patients in the United States and France had urine porphyrin levels associated with mercury toxicity. A prospective study of urinary porphyrin testing at LabCorp (United States) and the Laboratoire Philippe Auguste (France) involving 71 autism spectrum disorder (ASD) patients, neurotypical sibling controls, and general population controls was undertaken. ASD patients had significant elevations in urinary levels of cP, 5cxP, and prcP relative to controls, and > 50% of ASD patients had urinary cP levels more than 2 standard deviations above the mean values for neurotypical sibling controls. Significant reductions in urinary 5cxP and cP levels were observed in ASD patients following chelation. A significant correlation was found between urinary porphyrins measured at LabCorp and those measured at the Laboratoire Philippe Auguste on individual ASD patients. The established developmental neurotoxicity attributed to mercury and biochemical/genomic evidence for mercury susceptibility/toxicity in ASDs indicates a causal role for mercury. Urinary porphyrin testing is clinically available, relatively inexpensive, and noninvasive. Porphyrins need to be routinely measured in ASDs to establish if mercury toxicity is a causative factor and to evaluate the effectiveness of chelation therapy.

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David Geier has been a consultant in cases involving vaccines/biologics before the no-fault National Vaccine Injury Compensation Program and in civil litigation. Dr. Mark Geier has been an expert witness and consultant in cases involving vaccines/biologics before the no-fault National Vaccine Injury Compensation Program and in civil litigation. David and Mark Geier have a patent pending for the treatment of autistic disorders.

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Porphyrins are derivatives of the heme synthesis pathway that afford a measure of xenobiotic exposure (Brewster, 1988). Heme production primarily occurs in liver, kidneys, and erythroid cells. The synthetic process is summarized in Figure 1 (Nataf et al., 2006). Excess porphyrinogen metabolites are excreted in the urine as oxidized porphyrins, particularly uroporphyrin (uP) and coproporphyrin (cP), the most abundant soluble porphyrin molecules in the kidney cortex (Woods & Miller, 1993). Because these mid-pathway porphyrins are the most water-soluble of all the porphyrins, they are excreted predominantly in urine, whereas the hydrophobic protoporphyrin is predominantly found in the bile and feces.

Excess urinary porphyrin excretion, or porphyrinuria, results from inhibition of key enzymatic steps in conditions including genetic deficiencies in heme production enzymes (Sarkany, 1999), hepatitis, renal disease, and erythroid disease (Gross et al., 2000), as well as by heavy metal inhibition of heme enzyme synthesis (Woods, 1996). Both in experimental animals and in humans exposed to heavy metals, elevated levels of porphyrins are found in the urine (Bowers et al., 1992; Woods, 1996). The steps in the heme pathway most vulnerable to heavy metal inhibition are those in which uroporphyrin decarboxylase (UROD) (Woods & Kardish, 1983) and coproporphyrinogen oxidase (CPOX) (Woods et al., 2005) are involved. The result of these inhibitions is specific elevations of cP and pentacarboxyporphyrin (5cxP) in the urine. A causal relationship between heavy metal inhibition and porphyrinuria was demonstrated both in rats (Pingree et al., 2001) and humans exposed to mercury (Woods et al., 1993), as well as in humans exposed to lead (Rosen & Markowitz, 1993). Investigators also observed that heavy metal removal with chelating agents reduced urinary porphyrin levels to control values (Gonzalez-Ramirez et al., 1995). Although nonmetal agents

ORIGINAL ARTICLE

Neurodevelopmental Disorders, Maternal Rh-Negativity, and Rho(D) Immune Globulins: A Multi-Center Assessment

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Abstract

BACKGROUND: Many formulations of Thimerosal (49.55% mercury by weight)-containing Rho(D) immune globulins (TCRs) were routinely administered to Rh-negative mothers in the US prior to 2002.

OBJECTIVES: It was hypothesized: (1) if prenatal Rho(D)-immune globulin preparation exposure was a risk factor for neurodevelopmental disorders (NDs) then more children with NDs would have Rh-negative mothers compared to controls; and (2) if Thimerosal in the Rho(D)-immune globulin preparations was the ingredient associated with NDs, following the removal of Thimerosal from all manufactured Rho(D)-immune globulin preparations from 2002 in the US the frequency of maternal Rh-negativity among children with NDs should be similar to control populations.

METHODS: Maternal Rh-negativity was assessed at two sites (Clinic A-Lynchburg, VA; Clinic B-Rockville and Baltimore, MD) among 298 Caucasian children with NDs and known Rh-status. As controls, maternal Rh-negativity frequency was determined from 124 Caucasian children (born 1987–2001) without NDs at Clinic A, and the Rh-negativity frequency was determined from 1,021 Caucasian pregnant mothers that presented for prenatal genetic care at Clinic B (1980–1989). Additionally, 22 Caucasian patients with NDs born from 2002 onwards (Clinics A and B) were assessed for maternal Rh-negativity.

RESULTS: There were significant and comparable increases in maternal Rh-negativity among children with NDs (Clinic: A=24.2%), autism spectrum disorders (Clinic: A=28.3%, B=25.3%), and attention-deficit-disorder/attention-deficit-hyperactivity-disorder (Clinic: A=26.3%) observed at both clinics in comparison to both control groups (Clinic: A=12.1%, B=13.9%) employed. Children with NDs born post-2001 had a maternal Rh-negativity frequency (13.6%) similar to controls.

CONCLUSION: This study associates TCR exposure with some NDs in children.

Potential Conflict of Interest: David Geier has been a consultant in legal cases involving vaccines/biologics. Dr. Elizabeth Mumper and Dr. Mark Geier have been expert witnesses and consultants in legal cases involving vaccines/biologics.

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A Prospective Blinded Evaluation of Urinary Porphyrins Verses the Clinical Severity of Autism Spectrum Disorders

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A prospective, blinded study evaluated the relationship between autism spectrum disorder (ASD) severity measured by Childhood Autism Rating Scale (CARS) scores and urinary porphyrins among a cohort of participants (n = 26). LabCorp (CLIA-approved) tested for uroporphyrins, heptacarboxylporphyrins, hexacarboxylporphyrins, pentacarboxylporphyrins, coproporphyrin (cP) I, and cP III levels. Participants with severe ASD had significantly increased cP I, cP III, and total cP levels in comparison to participants with mild ASD. A significant correlation was observed between increasing cP levels and CARS scores. Significant correlations were also noted for comparative urinary porphyrin testing between LabCorp and the Laboratoire Philippe Auguste (ISO-approved) for total cP. Finally, total cP measured at LabCorp was found to significantly correlate with precoproporphryin (a specific porphyrin marker for mercury toxicity) measured at the Laboratoire Philippe Auguste. Since urinary porphyrin testing is clinically available, relatively inexpensive, and noninvasive, it may be used to help suggest whether heavy metal toxicity is associated with ASD.

Nataf et al. (2006) were the first investigators to describe elevations in specific urinary porphyrin metabolites in a cohort of subjects diagnosed with autism spectrum disorders (ASD). These investigators observed that urinary porphyrins, pentacarboxyporphyrin (5cxP), precoproporphyrin (prcP), and total coproporphyrins (cP) (I + III), which are associated with increased mercury (Hg) body burden, were significantly elevated in subjects diagnosed with autism (n = 106) relative to controls. In contrast, other urinary porphyrin metabolite levels were similar among subjects diagnosed with autism in comparison to controls. Further, these investigators observed that 5cxP, prcP, and total cP (I + III) were reported to increase across the ASD spectrum from mild to severe clinical symptoms (Asperger's disorder < pervasive developmental delay–not otherwise specified (PDD-NOS) < autism < autism + epilepsy), whereas other urinary porphyrin metabolites were not found to significantly fluctuate in correspondence with ASD severity. Finally, these investigators observed that *meso*-2,3-dimercaptosuccinic acid (DMSA)-based chelation therapy significantly decreased urinary prcP and total cP (I + III) levels in a cohort of subjects diagnosed with autism.

Subsequent studies on cohorts of subjects diagnosed with ASD in the United States by Geier and Geier (2006, 2007b), in France by Nataf et al. (2008), and in Australia by Austin and Shandley (2008) have revealed comparable results. In addition, another recent study by Geier et al. (2009) evaluated urinary porphyrin metabolites in a prospective, blinded cohort study of subjects diagnosed with ASD. The study evaluated ASD severity based upon Childhood Autism Rating Scale (CARS) scores calculated prior to blind laboratory testing for urinary porphyrins. It was observed that study subjects with a severe ASD diagnosis in comparison to study subjects with a mild ASD diagnosis had significantly increased urinary porphyrin levels of 5cxP, prcP, and total cP (I + III), whereas other urinary porphyrin levels were similar in both groups. In addition, regression analyses showed significant relationships between increasing CARS scores and rising urinary 5cxP and prcP levels. These correlations were absent for other urinary porphyrin metabolites examined. Finally, it was observed that increasing urinary 5cxP and prcP levels were significantly correlated with impaired glutathione detoxification (Geier et al., 2009).

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A prospective study of prenatal mercury exposure from maternal dental amalgams and autism severity

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Dental amalgams containing 50% mercury (Hg) have been used in dentistry for the last 150 years, and Hg exposure during key developmental periods was associated with autism spectrum disorders (ASDs). This study examined increased Hg exposure from maternal dental amalgams during pregnancy among 100 qualifying participants born between 1990–1999 and diagnosed with DSM-IV autism (severe) or ASD (mild). Logistic regression analysis (age, gender, race, and region of residency adjusted) by quintile of maternal dental amalgams during pregnancy revealed the ratio of autism:ASD (severe:mild) were about 1 (no effect) for ≤5 amalgams and increased for ≥6 amalgams. Subjects with ≥6 amalgams were 3.2-fold significantly more likely to be diagnosed with autism (severe), in comparison to ASD (mild), than subjects with ≤5 amalgams. Dental amalgam policies should consider Hg exposure in women before and during the child-bearing age and the possibility of subsequent fetal exposure and adverse outcomes.

Key words: Asperger's syndrome, autism, developmental delay, neurodevelopmental disorder

INTRODUCTION

The practice of using amalgams (which generally contain 50% mercury) in dentistry has existed for over 150 years. As of mid-2008, the US Food and Drug Administration (FDA) has declined to classify the medical-device safety of amalgams used in dentistry. The American Dental Association maintains that the mercury in amalgam is safe and that the mercury does not leak (Edlich et al. 2007).

Yet, the research evidence suggests that there is significant amount of elemental leaching and mercury vapor release from amalgams (Cohen and Penugonda 2001) and that this liberated mercury is absorbed by several body tissues (Mutter et al. 2004, Edlich et al. 2007). As a result, dental amalgams are a significant source of mercury body burden, as studies in animals and humans show (Mutter et al. 2007). For example, Guzzi and coworkers (2006) found that, on autopsy, total mercury levels were significantly higher in subjects with a greater number of amalgam surfaces (>12) compared with those who had fewer (0–3), in all types of tissue. These authors also reported that the greater the number of amalgams, the greater the likelihood that mercury would be found in the brain. In regard to amalgam bearers, other investigators have reported an approximate 2- to 5-fold increase of the mercury level in blood and urine as well as a 2- to 12-fold increase of the mercury concentration in several body tissues (Mutter et al. 2007). Also, mercury from maternal amalgam fillings leads to a significant increase of mercury concentration in the tissues and the hair of fetuses and newborn children. Furthermore, placental, fetal, and infant mercury body burden correlates with the numbers of amalgam fillings of the mothers (Mutter et al. 2007, Palkovicova et al. 2008). Finally, mercury levels in amniotic fluid and breast milk correlate significantly with the number of maternal dental amalgam fillings (Mutter et al. 2007).

The overall importance of dental amalgams, particularly maternal dental amalgams, significantly contributing to fetal and early infant mercury body-burden stems from the fact that recent studies have postulated that mercury exposure can cause immune, sensory,

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Mitochondrial dysfunction, impaired oxidative-reduction activity, degeneration, and death in human neuronal and fetal cells induced by low-level exposure to thimerosal and other metal compounds

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Thimerosal (ethylmercurithiosalicylic acid), an ethylmercury (EtHg)-releasing compound (49.55% mercury (Hg)), was used in a range of medical products for more than 70 years. Of particular recent concern, routine administering of Thimerosal-containing biologics/childhood vaccines have become significant sources of Hg exposure for some fetuses/infants. This study was undertaken to investigate cellular damage among *in vitro* human neuronal (SH-SY-5Y neuroblastoma and 1321N1 astrocytoma) and fetal (nontransformed) model systems using cell vitality assays and microscope-based digital image capture techniques to assess potential damage induced by Thimerosal and other metal compounds (aluminum (Al) sulfate, lead (Pb)(II) acetate, methylmercury (MeHg) hydroxide, and mercury (Hg)(II) chloride) where the cation was reported to exert adverse effects on developing cells. Thimerosal-associated cellular damage was also evaluated for similarity to pathophysiological findings observed in patients diagnosed with autistic disorders (ADs). Thimerosal-induced cellular damage as evidenced by concentration- and time-dependent mitochondrial damage, reduced oxidative–reduction activity, cellular degeneration, and cell death in the *in vitro* human neuronal and fetal model systems studied. Thimerosal at low nanomolar (nM) concentrations induced significant cellular toxicity in human neuronal and fetal cells. Thimerosal-induced cytoxicity is similar to that observed in AD pathophysiologic studies. Thimerosal was found to be significantly more toxic than the other metal compounds examined. Future studies need to be conducted to evaluate additional mechanisms underlying Thimerosal-induced cellular damage and assess potential co-exposures to other compounds that may increase or decrease Thimerosal-mediated toxicity.

Keywords: autism; glial; lead; mercury; mercuric; neurodevelopmental

Introduction

Thimerosal (ethylmercurithiosalicylic acid) is an ethylmercury (EtHg)-releasing compound that has been used in a range of medical products for more than 70 years (Geier et al. 2007). Thimerosal contains 49.55% mercury (Hg) and, in aqueous solutions, is

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Blood mercury levels in autism spectrum disorder: Is there a threshold level?

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Mercury (Hg) may significantly impact the pathogenesis of autism spectrum disorders (ASDs). Lab results generated by Vitamin Diagnostics (CLIA-approved), from 2003-2007, were examined among subjects diagnosed with an ASD (n=83) in comparison to neurotypical controls (n=89). Blood Hg levels were determined by analyzing Hg content in red blood cells (RBC) using cold vapor analysis, and consistent Hg measurements were observed between Vitamin Diagnostics and the University of Rochester. Adjusted (age, gender, and date of collection) mean Hg levels were 1.9-fold significantly (P<0.0001) increased among subjects diagnosed with an ASD (21.4 μg/L) in comparison to controls (11.4 μg/L). Further, an adjusted significant (P<0.0005) threshold effect (>15 μg/L) was observed for Hg levels on the risk of a subject being diagnosed with an ASD in comparison to controls (odds ratio=6.4). The weight of scientific evidence supports Hg as a causal factor in subjects diagnosed with an ASD.

Key words: Asperger, autistic, body-burden, neurodevelopmental, PDD

INTRODUCTION

Autism spectrum disorders (ASDs) are neurodevelopmental disorders, presenting in childhood that affect at least 1 in 110 children in the United States (Centers for Disease Control and Prevention 2009). The condition is characterized by severe impairments in socialization, communication, and behavior. Individuals diagnosed with an ASD may display a range of problem behaviors such as hyperactivity, poor attention, impulsivity, aggression, self-injury, and tantrums. Further, these children often display unusual responses to sensory stimuli, such as hypersensitivities to light, sound, color, smell or touch, and have a high threshold to pain (Austin 2008).

Emerging evidence supports the theory that some ASDs may result from a combination of genetic/biochemical susceptibility, specifically a reduced ability to excrete mercury (Hg), and exposure to Hg at critical developmental periods (Austin 2008, Geier et al. 2008, 2009e). Exposure to Hg can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining/associated with ASDs, and these similarities extend to neuroanatomy, neurotransmitters, and biochemistry (Austin 2008, Geier et al. 2008, 2009e).

DeSoto and Hitlan (2007) postulated that if Hg does play any causal role in facilitating an ASD diagnosis, there would likely be at least some correlation between high Hg measured in the blood and the symptoms of autism, even if an individual's ability to metabolize mercury mediates the relationship between exposure and neural toxicity. This is because even if exposure is identical, those who remove Hg less effectively should still have higher levels in the blood. Subsequently, these researchers analyzed blood Hg levels in a cohort of children from China (ASDs and controls). These researchers concluded that a statistically significant relationship exists between total blood Hg levels and a diagnosis of an ASD (DeSoto and Hitlan 2007).

A subsequent study by Hertz-Picciotto and coauthors (2010) examined blood Hg levels documented in

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The biological basis of autism spectrum disorders: Understanding causation and treatment by clinical geneticists

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Autism spectrum disorders (ASDs), also known as pervasive developmental disorders (PDD), are a behaviorally defined group of neurodevelopmental disorders that are usually diagnosed in early childhood. ASDs disproportionately affect male children. Mercury (Hg), a heavy metal, is widespread and persistent in the environment. Mercury is a ubiquitous source of danger in fish, drugs, fungicides/herbicides, dental fillings, thermometers, and many other products. Elevated Hg concentrations may remain in the brain from several years to decades following exposure. This is important because investigators have long recognized that Hg is a neurodevelopmental poison; it can cause problems in neuronal cell migration and division, and can ultimately cause cell degeneration and death. Case-reports of patients have described developmental regressions with ASD symptoms following fetal and/or early childhood Hg exposure, and epidemiological studies have linked exposure to Hg with an elevated risk of a patient being diagnosed with an ASD. Immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with ASDs were reported following Hg intoxication with similarities extending to neuroanatomy, neurotransmitters, and biochemistry. The sexual dimorphism of ASDs may result from synergistic neurotoxicity caused by the interaction of testosterone and Hg; in contrast, estrogen is protective, mitigating the toxicity of Hg. Mercury exposure may significantly increase androgen levels, and as a result, patients diagnosed with an ASD may significantly benefit from anti-androgen therapy. Finally, the clinical geneticist has a wealth of biomarkers to evaluate and treat patients diagnosed with an ASD.

Key words: autistic, estradiol, ethylmercury, merthiolate, methylmercury, Thimerosal

INTRODUCTION

Autism spectrum disorders (ASDs), also known as pervasive developmental disorders (PDD), are a behaviorally defined group of neurodevelopmental disorders that are usually diagnosed in early childhood. ASDs disproportionately affect male children (roughly, 5 males per 1 female) (Austin 2008). ASDs are characterized by early onset of impairments in social interaction and communication, and the development of unusual stereotyped behaviors. Unable to learn from the natural environment as most children, the child diagnosed with an ASD generally shows little interest in the world or people around him/her. Although a few children with an ASD develop normal and even advanced skills in particular areas, most exhibit a wide range of profound behavioral problems and delayed or undeveloped skills. Further, a child diagnosed with an ASD may display a range of problem behaviors such as hyperactivity, poor attention, impulsivity, aggression, self injury and tantrums. In addition, many frequently display unusual responses to sensory stimuli such as hypersensitivities to light or certain sounds, colors, smells, or touch and have a high threshold of pain (Austin 2008). Further, common co-morbidity conditions often associated with an ASD diagnosis include gastrointestinal disease and dysbiosis (White 2003), autoimmune disease (Sweeten et al. 2003), and mental retardation (Bolte and Poustka 2002). Therefore, in the absence of treatment, an ASD is, in general, a lifelong developmental disability that profoundly affects the way a person comprehends, communicates and relates to others.

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Translational Neurodegeneration

RESEARCH Open Access

A two-phase study evaluating the relationship between Thimerosal-containing vaccine administration and the risk for an autism spectrum disorder diagnosis in the United States

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Abstract

Background: Autism spectrum disorder (ASD) is defined by standardized criteria of qualitative impairments in social interaction, qualitative impairments in communication, and restricted and stereotyped patterns of behavior, interests, and activities. A significant number of children diagnosed with ASD suffer a loss of previously-acquired skills, which is suggestive of neurodegeneration or a type of progressive encephalopathy with an etiological pathogenic basis occurring after birth. To date, the etiology of ASD remains under debate, however, many studies suggest toxicity, especially from mercury (Hg), in individuals diagnosed with an ASD. The present study evaluated concerns about the toxic effects of organic-Hg exposure from Thimerosal (49.55% Hg by weight) in childhood vaccines by conducting a two-phased (hypothesis generating/hypothesis testing) study with documented exposure to varying levels of Thimerosal from vaccinations.

Methods: A hypothesis generating cohort study was undertaken to evaluate the relationship between exposure to organic-Hg from a Thimerosal-containing Diphtheria-Tetanus-acellular-Pertussis (DTaP) vaccine in comparison to a Thimerosal-free DTaP vaccine administered, from 1998 through 2000, for the risk of ASD as reported in the Vaccine Adverse Event Reporting System (VAERS) database (phase I). A hypothesis testing case–control study was undertaken to evaluate the relationship between organic-Hg exposure from Thimerosal-containing hepatitis B vaccines administered at specific intervals in the first six months of life among cases diagnosed with an ASD and controls born between 1991 through 1999 in the Vaccine Safety Datalink (VSD) database (phase II).

Results: In phase I, it was observed that there was a significantly increased risk ratio for the incidence of ASD reported following the Thimerosal-containing DTaP vaccine in comparison to the Thimerosal-free DTaP vaccine. In phase II, it was observed that cases diagnosed with an ASD were significantly more likely than controls to receive increased organic-Hg from Thimerosal-containing hepatitis B vaccine administered within the first, second, and sixth month of life.

Conclusions: Routine childhood vaccination is an important public health tool to reduce the morbidity and mortality associated with infectious diseases, but the present study provides new epidemiological evidence supporting an association between increasing organic-Hg exposure from Thimerosal-containing childhood vaccines and the subsequent risk of an ASD diagnosis.

Keywords: Autism, Ethylmercury, Merthiolate, Thimerosal, Thiomersal, Vaccine

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Research Article

The risk of neurodevelopmental disorders following a Thimerosal-preserved DTaP formulation in comparison to its Thimerosal-reduced formulation in the vaccine adverse event reporting system (VAERS)

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Abstract: Mercury (Hg) exposure in human infants and fetuses has long been known to be significantly associated with neurodevelopmental disorders (NDs). Thimerosal (49.55% Hg by weight) is an ethyl-Hg containing compound added to many childhood vaccines as a preservative. A hypothesis testing case-control study was undertaken in the Vaccine Adverse Event Reporting System (VAERS) database (updated through September 2013) by examining 5,591 adverse event reports entered following Thimerosal-preserved Diphtheria-Tetanus-acellular-Pertussis (DTaP) (Tripedia™, Sanofi) administered from 1997-1999 (exposed) and following Thimerosal-reduced DTaP (Tripedia™, Sanofi) administered from 2004-2006 (unexposed). Cases were defined as individuals with adverse event reports with the outcomes of autism, speech disorder, mental retardation, or ND (at least of one these aforementioned specific outcomes being mentioned in the adverse event report). Controls were defined as individuals with adverse event reports without any mention of the specific case outcomes examined. Cases reported with the outcomes of autism (odds ratio = 7.67, $p < 0.0001$), speech disorders (odds ratio = 3.49, $p < 0.02$), mental retardation (odds ratio = 8.73, $p < 0.0005$), or ND (odds ratio = 4.82, $p < 0.0001$) were significantly more likely than controls to have received Thimerosal-preserved DTaP vaccine (exposed) in comparison to Thimerosal-reduced DTaP vaccine (unexposed). Though routine childhood vaccination is considered an important public health tool to reduce the morbidity and mortality associated with certain infectious diseases, this study supports a significant relationship between increased organic-Hg exposure from Thimerosal-preserved childhood vaccines and the child's subsequent risk of a ND diagnosis.

Keywords: autistic, developmental delay, ethylmercury, merthiolate, language, thiomersal

Introduction

Mercury (Hg) is a heavy metal that is widespread and persistent in the environment, and infants in the US are exposed to significant levels of environmental Hg through air, water, and breast milk [1]. In addition to environmental Hg exposure and maternal exposures from the mother's Hg body burden, dietary intakes, and Hg-containing pharmaceuticals administered to the mother while the child is developing in utero, and injected organic-Hg from

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A Case-Control Study Evaluating the Relationship Between Thimerosal-Containing *Haemophilus influenzae* Type b Vaccine Administration and the Risk for a Pervasive Developmental Disorder Diagnosis in the United States

David A. Geier · Janet K. Kern · Paul G. King · Lisa K. Sykes · Mark R. Geier

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Abstract Thimerosal is an organic mercury (Hg)-containing compound (49.55 % Hg by weight) historically added to many multi-dose vials of vaccine as a preservative. A hypothesis testing case-control study evaluated automated medical records in the Vaccine Safety Datalink (VSD) for organic Hg exposure from Thimerosal in *Haemophilus influenzae* type b (Hib)-containing vaccines administered at specific times within the first 15 months of life among subjects diagnosed with pervasive developmental disorder (PDD) (n=534) in comparison to controls. The generally accepted biologically non-plausible linkage between Thimerosal exposure and subsequent diagnosis of febrile seizure (n=5886) was examined as a control outcome. Cases diagnosed with PDD received significantly more organic Hg within the first 6 months of life (odds ratio (OR)=1.97, p<0.001) and first 15 months of life (OR=3.94, p<0.0001) than controls, whereas cases diagnosed with febrile seizure were no more likely than controls to have received increased organic Hg. On a per microgram of organic Hg basis, cases diagnosed with a PDD in comparison to controls were at significantly greater odds (OR=1.0197, p<0.0001) of receiving increasing organic Hg exposure within the first 15 months of life, whereas cases diagnosed febrile seizure were no more likely than controls (OR=0.999, p>0.20) to have received increasing organic Hg exposure within the first 15 months of life. Routine childhood vaccination is an important public health tool to reduce the morbidity and mortality associated with infectious diseases, but the present study provides new epidemiological evidence of a significant relationship between increasing organic Hg exposure from Thimerosal-containing vaccines and the subsequent risk of PDD diagnosis in males and females.

Keywords Autism · Ethylmercury · Merthiolate · Thimerosal · Thiomersal · Vaccine

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Introduction

Thimerosal is an organic mercury (Hg)-containing compound (49.55 % Hg by weight) historically added to many multi-dose vials of vaccine as a preservative since the 1930s [1]. Thimerosal is initially metabolized into ethyl-Hg compounds and thiosalicylate and rapidly binds onto thiol groups found on many proteins in human blood [2]. It is then actively transported across the blood brain barrier, including by the L-type neutral amino acid carrier transport (LAT) system, into human neuronal cells [3, 4], where it significantly accumulates and persists for many months following exposure and alters numbers of neurons in the dentate gyrus of the hippocampus and thalamus [5, 6].

In 2008, an ecological birth cohort assessment of Thimerosal exposure in infants and neurodevelopment disorders within the computerized medical records of the Vaccine Safety Datalink (VSD) database was undertaken [7]. A total of 278,624 subjects were examined in birth cohorts from 1990–1996 that had received their first oral polio vaccination by 3 months of age in the VSD. The birth cohort prevalence rate of the medically diagnosed pervasive developmental disorders (PDDs) of autism and autism spectrum disorder (ASD) was

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Article

A Dose-Response Relationship between Organic Mercury Exposure from Thimerosal-Containing Vaccines and Neurodevelopmental Disorders

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Abstract: A hypothesis testing case-control study evaluated concerns about the toxic effects of organic-mercury (Hg) exposure from thimerosal-containing (49.55% Hg by weight) vaccines on the risk of neurodevelopmental disorders (NDs). Automated medical records were examined to identify cases and controls enrolled from their date-of-birth (1991–2000) in the Vaccine Safety Datalink (VSD) project. ND cases were diagnosed with pervasive developmental disorder (PDD), specific developmental delay, tic disorder or hyperkinetic syndrome of childhood. In addition, putative non-thimerosal-related outcomes of febrile seizure, failure to thrive and cerebral degenerations were examined. The cumulative total dose of Hg exposure from thimerosal-containing hepatitis B vaccine (T-HBV) administered within the first six months of life was calculated. On a per microgram of organic-Hg basis, PDD (odds ratio (OR) = 1.054), specific developmental delay (OR = 1.035), tic disorder (OR = 1.034) and hyperkinetic syndrome of childhood (OR = 1.05) cases were significantly more likely than controls to receive increased organic-Hg exposure. By contrast, none of the non-thimerosal related outcomes were significantly more likely than the controls to have received increased organic-Hg exposure. Routine childhood vaccination may be an important public health tool to reduce infectious disease-associated morbidity/mortality, but the present study significantly associates organic-Hg exposure from T-HBV with an increased risk of an ND diagnosis.

Keywords: attention deficit; autism; ethylmercury; merthiolate; thiomersal

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ORIGINAL ARTICLE

Thimerosal exposure and increased risk for diagnosed tic disorder in the United States: a case-control study

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ABSTRACT

A hypothesis testing, case-control study evaluated automated medical records for exposure to organic-Hg from Thimerosal-containing hepatitis B vaccines (TM-HepB) administered at specific intervals in the first six-months-of-life among cases diagnosed with a tic disorder (TD) or cerebral degeneration (CD) (an outcome not biologically plausibly linked to TM exposure) in comparison to controls; both cases and controls were continuously enrolled from birth (born from 1991–2000) within the Vaccine Safety Datalink (VSD) database. TD cases were significantly more likely than controls to have received increased organic-Hg from TM-HepB administered within the first month-of-life (odds ratio (OR)=1.59, $p<0.00001$), first two-months-of-life (OR=1.59, $p<0.00001$), and first six-months-of-life (OR=2.97, $p<0.00001$). Male TD cases were significantly more likely than male controls to have received increased organic-Hg from TM-HepB administered within the first month-of-life (OR =1.65, $p<0.0001$), first two-months-of-life (OR=1.64, $p<0.0001$), and first six months-of-life (OR=2.47, $p<0.05$), where as female TD were significantly more likely than female controls to have received increased organic-Hg from TM-HepB administered within the first six-months-of-life (OR=4.97, $p<0.05$). By contrast, CD cases were no more likely than controls to have received increased organic-Hg exposure from TM-HepB administered at any period studied within the first six-months-of-life. Although routine childhood vaccination is considered an important public health tool to combat infectious diseases, the present study associates increasing organic-Hg exposure from TM-HepB and the subsequent risk of a TD diagnosis.

KEY WORDS: ethylmercury, merthiolate, thiomersal, tic, tourette, vaccine

Introduction

Tic disorder (TD) is a neurodevelopmental disorder characterized by repetitive, involuntary movements and vocalizations called tics (Diagnostic and Statistical Manual of Mental Disorders – Fifth Edition; DSM-5, 2013; Roessner *et al.,* 2011). TD includes Tourette's syndrome, which is characterized by vocal as well as motor tics. Symptoms of TD typically begin in childhood, with the average onset between 3 and 9 years of age. Males are affected approximately three to four times more often than females. TD is considered a chronic condition that lasts a lifetime (National Institute of Neurological Disorders and Stroke, 2012). Psychopathology and co-morbidity occur in approximately 80–90% of clinical cohorts (Hariz *et al.,* 2010). Two of the most common co-occurring psychiatric conditions are: (1) attention deficit/hyperactivity disorder (ADHD), occurring in about half the cases (Roessner *et al.,* 2011; Freeman 2007) and (2) obsessive–compulsive disorder (OCD), also occurring in about half the cases (Roessner *et al.,* 2011). Other common co-morbid conditions are depression, anxiety, and behavioral disorders (Hariz *et al.,* 2010). Also reported are social difficulties and ritualistic behaviors such as counting, repeating, ordering, and arranging. Along with the dramatic rise in neurodevelopmental disorders in general in the last two decades, there has also been an increase in TD (Boyle *et al.,* 2011; Cubo 2012). Although TD was once considered rare, today TD is considered the most common movement disorder, with 0.2–46.3% of schoolchildren experiencing tics during their lifetime (Cubo, 2012). To date, there is no consensus on the causes or contributing factors related to this increase. Many questions regarding the potential

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Invited critical review

Thimerosal: Clinical, epidemiologic and biochemical studies ☆

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ABSTRACT

Introduction: Thimerosal (or Thiomersal) is a trade name for an organomercurial compound (sodium ethylmercury (Hg) thiosalicylate) that is 49.55% Hg by weight, which rapidly decomposes in aqueous saline solutions into ethyl-Hg hydroxide and ethyl-Hg chloride. Developed in 1927, it has been and is still being used as a preservative in some cosmetics, topical pharmaceuticals, and biological drug products, including vaccines. Concerns have been voiced about its use because it is toxic to human cells. Although it is banned in several countries, it continues to be added to some vaccines in the United States and many vaccines in the developing world.

Discussion: This critical review focuses on the clinical, epidemiological, and biochemical studies of adverse effects from Thimerosal in developing humans. This review will include research that examines fetal, infant, and childhood death; birth defects; neurodevelopmental testing deficits in children; and neurodevelopmental disorders (attention deficit/hyperactivity disorder, autism spectrum disorder, tic disorder, and specific developmental delays). The review will also look at the research that examined the outcomes of acute accidental ethyl-Hg poisoning in humans. The studies that examine the underlying biochemical insights into the neuronal cellular damage will also be explored.

Conclusion: The culmination of the research that examines the effects of Thimerosal in humans indicates that it is a poison at minute levels with a plethora of deleterious consequences, even at the levels currently administered in vaccines.

Contents

☆ Conflict of interest: Six (6) of authors have been involved in vaccine/biologic legal actions (DAG, PGK, BSH, JKK, LSK, and MRG). One author (JGD) has no conflict of interest.

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ORIGINAL ARTICLE

A longitudinal cohort study of the relationship between Thimerosal-containing hepatitis B vaccination and specific delays in development in the United States: Assessment of attributable risk and lifetime care costs

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KEYWORDS
Ethylmercury;
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Vaccine

Abstract Epidemiological evidence suggests a link between mercury (Hg) exposure from Thimerosal-containing vaccines and specific delays in development. A hypothesis-testing longitudinal cohort study (n = 49,835) using medical records in the Vaccine Safety Datalink (VSD) was undertaken to evaluate the relationship between exposure to Hg from Thimerosal-containing hepatitis B vaccines (T-HBVs) administered at specific intervals in the first 6 months of life and specific delays in development [International Classification of Disease, 9th revision (ICD-9): 315.xx] among children born between 1991 and 1994 and continuously enrolled from birth for at least 5.81 years. Infants receiving increased Hg doses from T-HBVs administered within the first month, the first 2 months, and the first 6 months of life were significantly more likely to be diagnosed with specific delays in development than infants receiving no Hg doses from T-HBVs. During the decade in which T-HBVs were routinely recommended and administered to US infants (1991–2001),

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A Two-Phase Case-Control Study of Autism Risk Among Children Born From the Late 1990s Through the Early 2000s in the United States

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: This study was supported by the non-profit group CoMeD, Inc. and by the non-profit Institute of Chronic Illnesses, Inc.

Background: This study evaluated the hypothesis that the 1999 recommendation by the American Academy of Pediatrics (AAP) and US Public Health Service (PHS) to reduce exposure to mercury (Hg) from Thimerosal in US vaccines would be associated with a reduction in the long-term risk of being diagnosed with autism.

Material/Methods: A two-phase assessment utilizing a case (n=73) -control (n=11,783) study in the Vaccine Adverse Event Reporting System (VAERS) database (for hypothesis generating) and a more rigorous, independent matched case (n=40) -control (n=40) study (hypothesis testing) was undertaken.

Results: Analysis of the VAERS database using logistic regression revealed that the odds ratio (OR) for being an autism case in the VAERS database significantly decreased with a more recent year of vaccination in comparison to controls (OR=0.65) from 1998 to 2003. Sex-separated analyses revealed similar significant effects for males (OR=0.62) and females (OR=0.71). Analyses of the matched case-control data revealed, using the t-test statistic, that the mean date of birth among cases diagnosed with an autism spectrum disorder (ASD) (2000.5±1.2) was significantly more in the past than in controls (2001.1±1.3). Logistic regression also revealed that the OR for being diagnosed with ASD significantly decreased with a more recent date of birth in comparison to controls (OR=0.67) from 1998–2003.

Conclusions: This study reveals that the risk of autism during from the late1990s to early 2000s in the US significantly decreased with reductions in Hg exposure from Thimerosal-containing childhood vaccines, but future studies should examine this phenomenon in other US populations. Vaccine programs have significantly reduced the morbidity and mortality associated with infectious disease, but Thimerosal should be removed from all vaccines.

MeSH Keywords: Autistic Disorder • Child Development Disorders, Pervasive • Ethylmercury Compounds • Thimerosal

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Thimerosal-Containing Hepatitis B Vaccination and the Risk for Diagnosed Specific Delays in Development in the United States: A Case-Control Study in the Vaccine Safety Datalink

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Abstract

Background:

Within the first 3 years of life, the brain develops rapidly. Its development is characterized by critical developmental periods for speech, vision, hearing, language, balance, etc.; and alteration in any of the processes occurring in those critical periods can lead to specific delays in development.

Aims:

The present study evaluated the potential toxic effects of organic-mercury exposure from Thimerosal (49.55% mercury by weight) in childhood vaccines and its hypothesized possible relationship with specific delays in development.

Materials and Methods:

A hypothesis testing case-control study was undertaken to evaluate the relationship between exposure to Thimerosal-containing hepatitis B vaccines administered at specific intervals in the first 6 months among cases diagnosed with specific delays in development and controls born between 1991-2000, utilizing data in the Vaccine Safety Datalink database.

Results:

Cases were significantly more likely than controls to have received increased organic-mercury from

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Thimerosal-containing Hepatitis B Vaccine Exposure is Highly Associated with Childhood Obesity: A Case-control Study Using the Vaccine Safety Datalink

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Abstract

Background:

Obesity among children and adolescents in the United States has tripled since 1980, and has become a major public health concern.

Aims:

The purpose of this study was to evaluate the potential relationship between exposure to organic mercury from Thimerosal-containing hepatitis B vaccines and the children's subsequent risk of an obesity diagnosis.

Materials and Methods:

A hypothesis-testing, case-control study was undertaken to evaluate exposure to organic mercury from Thimerosal-containing hepatitis B vaccines, which were administered at specific intervals in the first 6 months of life, among cases diagnosed with childhood obesity and controls by examining automated medical records for children born from 1991 to 2000 who were continuously enrolled in the Vaccine Safety Datalink database.

Results:

This study found highly significant associations as follows. Cases diagnosed with obesity were

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Article

Thimerosal-Preserved Hepatitis B Vaccine and Hyperkinetic Syndrome of Childhood

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Abstract: (1) Background: Hyperkinetic syndrome of childhood (HKSoC) is an International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9) category in which the majority of the children are also diagnosed under the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR), where the umbrella term is "Attention-Deficit and Disruptive Behavior Disorders". The diagnostic criteria for HKSoC are developmentally inappropriate inattention, hyperactivity, and impulsivity. Some studies have implicated mercury (Hg) exposure as a risk factor. (2) Methods: This hypothesis testing study; using the Vaccine Safety Datalink; assessed the toxicological effects of bolus exposure to organic-Hg from Thimerosal-containing vaccines (TCVs) by examining the relationship between Thimerosal-preserved hepatitis B vaccines (TM-HepB) given at varying levels and at specific intervals in the first six months after birth and the risk of a child being diagnosed with HKSoC. (3) Results: Children diagnosed with HKSoC were significantly more likely to be exposed to increased organic-Hg from TM-HepB doses given within the first month (odds ratio = 1.45; 95% confidence interval (CI) = 1.30–1.62); within the first two months (odds ratio = 1.43; 95% CI = 1.28–1.59); and within the first six months (odds ratio = 4.51; 95% CI = 3.04–6.71) than controls. (4) Conclusion: The results indicate that increasing organic-Hg exposure from TCVs heightens the risk of a HKSoC diagnosis.

Keywords: ADHD; Thimerosal; neurodevelopmental disorder; Hyperkinetic syndrome of childhood

1. Introduction

Hyperkinetic syndrome of childhood (HKSoC) is behaviorally defined in the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9) [1]. HKSoC is an ICD-9 category (314.xx) for the group of conditions, which include the following six subtypes: attention deficit disorder (ADD) without mention of hyperactivity (314.00); attention deficit disorder with hyperactivity (ADHD) (314.01); hyperkinesis with developmental delay (314.1); hyperkinetic conduct disorder (314.2); other specified manifestations of hyperkinetic syndrome (314.8); and unspecified hyperkinetic syndrome (314.9). The specific diagnostic criteria are features of developmentally inappropriate inattention, hyperactivity, and impulsivity [2]. The disorder is characterized by a marked pattern of inattention and/or hyperactivity-impulsivity that is inconsistent with developmental level and clearly interferes with normal functioning in at least two settings (e.g., at home and at school). At least some of the symptoms must be present before the age of seven years. Most children in this category are labeled with the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision

Abstract

Background: Thimerosal is an organic-mercury (Hg)-containing compound (49.55% Hg by weight) historically added to many multi-dose vials of vaccine as a preservative and still added to some vaccines today. Concerns about the toxic effects from Thimerosal-containing childhood vaccines and the risk of an atypical autism diagnosis were evaluated in this study.

Methods: A hypothesis-testing, prospective longitudinal, case-control study assessed exposure to Hg from Thimerosal-containing hepatitis B vaccines (TM-HepB) among cases diagnosed with atypical autism (n=164) and controls (n=15,216). Automated medical records for subjects born from 1991-2000 and continuously enrolled in the Vaccine Safety Datalink (VSD) database were examined.

Results: Cases diagnosed with atypical autism were statistically significantly more likely to have received greater overall and dose-dependent exposures to Hg from TM-HepB vaccines administered within the first month of life, first two months of life, and first six months of life than the controls. Similar phenomena were observed when cases and controls were separated by gender.

Conclusions: Routine childhood vaccination is an important public health tool to reduce infectious diseases. The present study provides important epidemiological evidence significantly associating increasing Hg exposure from Thimerosal-containing childhood vaccines and the subsequent risk of atypical autism diagnosis, and suggests that Thimerosal should be eliminated from vaccines.

Keywords: Asperger's disorder; Ethylmercury; Merthiolate; PDD-NOSl; Thiomersal; atypical autism

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Abnormal Brain Connectivity Spectrum Disorders Following Thimerosal Administration: A Prospective Longitudinal Case–Control Assessment of Medical Records in the Vaccine Safety Datalink

David A. Geier[1,2], Janet K. Kern[1,2,3], Kristin G. Homme[4], and Mark R. Geier[1,2]

Abstract

Background: Autism spectrum disorder (ASD), tic disorder (TD), and hyperkinetic syndrome of childhood (attention deficit disorder [ADD]/attention deficit hyperactivity disorder [ADHD]) are disorders recently defined as abnormal connectivity spectrum disorders (ACSDs) because they show a similar pattern of abnormal brain connectivity. This study examines whether these disorders are associated with exposure to thimerosal, a mercury (Hg)-based preservative.

Methods: A hypothesis testing case-control study evaluated the Vaccine Safety Datalink for the potential dose-dependent odds ratios (ORs) for diagnoses of ASD, TD, and ADD/ADHD compared to controls, following exposure to Hg from thimerosal-containing *Haemophilus influenzae* type b vaccines administrated within the first 15 months of life. Febrile seizures, cerebral degeneration, and unspecified disorders of metabolism, which are not biologically plausibly linked to thimerosal, were examined as control outcomes.

Results: On a per 25 μg Hg basis, cases diagnosed with ASD (OR = 1.493), TD (OR = 1.428), or ADD/ADHD (OR = 1.503) were significantly ($P < .001$) more likely than controls to have received increased Hg exposure. Similar relationships were observed when separated by gender. Cases diagnosed with control outcomes were no more likely than controls to have received increased Hg exposure.

Conclusion: The results suggest that Hg exposure from thimerosal is significantly associated with the ACSDs of ASD, TD, and ADD/ADHD.

Keywords
Asperger, autism, ethylmercury, PDD-NOS, thimerosal, Tourette, ADD/ADHD, Mercury

Introduction

Autism spectrum disorder (ASD), tic disorder (TD), and hyperkinetic syndrome of childhood (also known as attention deficit disorder [ADD]/attention deficit hyperactivity disorder [ADHD]) are neurodevelopmental disorders.[1] Evidence suggests these children share similar neuropathology, symptomatology, and comorbid conditions. Moreover, these disorders present with a similar pattern of abnormal brain connectivity of long-range underconnectivity and short-range overconnectivity.[1] As a consequence, it was suggested that these disorders may be subsets in what could be termed an abnormal connectivity spectrum disorder (ACSD).

It has also been hypothesized that the etiological basis of ACSDs is plausibly related to neuronal insult (eg,

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ORIGINAL ARTICLE

Thimerosal exposure and disturbance of emotions specific to childhood and adolescence: A case-control study in the Vaccine Safety Datalink (VSD) database

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ABSTRACT

Background: This study evaluated Thimerosal-containing childhood vaccines and the risk of a diagnosis called disturbance of emotions specific to childhood and adolescence (ED). Thimerosal is an organic-mercury (Hg)-containing compound used in some vaccines.

Methods: A hypothesis-testing prospective, longitudinal case-control study evaluated Hg exposure from Thimerosal in hepatitis B vaccines administered at specific times within the first 6 months of life and its association with medically diagnosed ED (313.xx) (n = 517) in children born between 1991–2000 in comparison to controls (n = 27 491) in the Vaccine Safety Datalink (VSD) database.

Results: Cases diagnosed with ED were significantly more likely than controls to have received increased Hg exposure within the first month of life (odds ratio (OR) = 1.3384), the first 2 months of life (OR = 1.3367) and the first 6 months of life (OR = 2.37). When the data were separated by gender, similar significant adverse effects were observed for males, but not females. On a per microgram Hg basis, cases diagnosed with ED were significantly more likely than controls to have received increased exposure within the first 6 months of life (OR = 1.025 per microgram Hg).

Conclusions: The results show a significant relationship between Hg exposure from Thimerosal-containing childhood vaccines and the subsequent risk of an ED diagnosis.

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KEYWORDS
Emotional disturbances; anxiety; ethylmercury; merthiolate; shyness; thiomersal; mercury; social impairment

Introduction

The International Classification of Diseases, Ninth Revision (ICD-9) code 313.xx is entitled, 'Disturbance of emotions specific to childhood and adolescence' (emotional disturbances (ED)). The following comprise the different types of ED diagnoses: (1) over-anxious disorder specific to childhood and adolescence; (2) misery and unhappiness disorder specific to childhood and adolescence; (3) shyness disorder of childhood; (4) introverted disorder of childhood; (5) selective mutism; (6) relationship problems specific to childhood and adolescence; (7) oppositional defiance disorder; (8) identity disorder of childhood or adolescence; (9) academic underachievement disorder of childhood or adolescence; (10) other emotional disturbances of childhood or adolescence; and (11) unspecified emotional disturbance of childhood or adolescence [1].

According to a 2005 analysis, as of 2004, there were ~ 450 000 students diagnosed with an ED in the US population, ~ 9% of all students [2]. They also reported that over 75% of youth classified as having an ED diagnosis were boys, and there was a wide range of co-morbid diagnoses, including anxiety, bipolar disorder, depression, oppositional behaviour, psychosis, attention deficit hyperactivity disorder (ADHD) and learning disability (LD). Approximately two-thirds had a co-morbid diagnosis of ADHD and approximately one-fourth had a co-morbid diagnosis of LD. In addition, children diagnosed with an ED have poorer societal outcomes than the general population and have high rates of criminal justice involvement [3].

At the present time there is no consensus on the cause of ED. Investigators previously described that interacting genetic, environmental and social factors are important determinants. Mercury (Hg) is a neurodevelopmental toxicant and extensive laboratory and clinical studies of Hg demonstrate the unique vulnerability of the developing brain to Hg [4].

In considering the sources of Hg exposure to infants, Thimerosal (sodium ethyl-Hg thiosalicylate, $C_9H_9HgNaO_2S$) is an ethylmercury-containing compound (49.55% Hg by weight) that was and continues to be utilized in vaccines that are routinely administered to pregnant women and infants in the US and worldwide [5].

In the US, prenatal exposure to Hg results from the routine recommendation to administer influenza vaccines to pregnant women at any time during pregnancy [5]. In addition, postnatal exposure to Hg results from the routine recommendation to administer three doses of influenza vaccine during the first 18 months of life, and then throughout childhood on an annual basis [5]. It is estimated that about half of the doses of influenza vaccine in the US still contain Thimerosal [5]. Worldwide, and in particular in developing nations, Thimerosal is still present in many vaccines routinely administered to infants/children [5]. It was previously estimated that ~ 50% of the Hg dose that some infants receive is from Thimerosal-

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Neurodevelopmental Disorders after Thimerosal-Containing Vaccines: A Brief Communication

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We were initially highly skeptical that differences in the concentrations of thimerosal in vaccines would have any effect on the incidence rate of neurodevelopmental disorders after childhood immunization. This study presents the first epidemiologic evidence, based upon tens of millions of doses of vaccine administered in the United States, that associates increasing thimerosal from vaccines with neurodevelopmental disorders. Specifically, an analysis of the Vaccine Adverse Events Reporting System (VAERS) database showed statistical increases in the incidence rate of autism (relative risk [RR] = 6.0), mental retardation (RR = 6.1), and speech disorders (RR = 2.2) after thimerosal-containing diphtheria, tetanus, and acellular pertussis (DTaP) vaccines in comparison with thimerosal-free DTaP vaccines. The male/female ratio indicated that autism (17) and speech disorders (2.3) were reported more in males than females after thimerosal-containing DTaP vaccines, whereas mental retardation (1.2) was more evenly reported among male and female vaccine recipients. Controls were employed to determine if biases were present in the data, but none were found. It was determined that overall adverse reactions were reported in similar-aged populations after thimerosal-containing DTaP (2.4 ± 3.2 years old) and thimerosal-free DTaP (2.1 ± 2.8 years old) vaccinations. Acute control adverse reactions such as deaths (RR = 1.0), vasculitis (RR = 1.2), seizures (RR = 1.6), ED visits (RR = 1.4), total adverse reactions (RR = 1.4), and gastroenteritis (RR = 1.1) were reported similarly after thimerosal-containing and thimerosal-free DTaP vaccines. An association between neurodevelopmental disorders and thimerosal-containing DTaP vaccines was found, but additional studies should be conducted to confirm and extend this study. Exp Biol Med 228:660–664, 2003

Key words: autism; neurodevelopmental disorders; thimerosal; VAERS

In recent years, thimerosal, an organic mercury compound that is metabolized to ethylmercury and thiosalicylate and has been present since the 1930s as a preservative in some vaccines and pharmaceutical products to prevent bacterial and fungal contamination, has come under scrutiny. It was determined by the U.S. Food and Drug Administration (FDA) in 1999 under the recommended childhood immunization schedule that infants might be exposed to cumulative doses of ethylmercury that exceed some federal safety guidelines established for exposure to methylmercury, another form of organic mercury (1).

The hypothesis that exposure to thimerosal-containing vaccines could be associated with neurodevelopmental disorders is not established and rests on indirect and incomplete information, primarily from analogies with methylmercury and levels of maximum mercury exposure from vaccines given in children. The hypothesis is biologically possible, but the possible relationship between thimerosal from vaccines and neurodevelopmental disorders of autism, attention deficit/hyperactivity disorder (ADHD), and speech or language delay remains seriously suspect. As of the present, there are no peer-reviewed epidemiological studies in the scientific literature examining the potential association between thimerosal-containing vaccines and neurodevelopmental disorders. Here, we show the first epidemiologic evidence, based upon tens of millions of doses of vaccine administered in the United States, that associates increasing thimerosal from vaccines with neurodevelopmental disorders.

Materials and Methods

In this study, the incidence of neurodevelopmental disorders in a comparative examination between thimerosal-containing diphtheria, tetanus, and acellular pertussis (DTaP) and thimerosal-free DTaP vaccines based upon analysis of the Vaccine Adverse Events Reporting System (VAERS) database was undertaken using Microsoft Access. The VAERS database is an epidemiologic database maintained by the Centers for Disease Control and Prevention (CDC) since 1990. All adverse reactions are to be reported to the VAERS database as required by U.S. law. The CDC requires written and telephonic confirmation of serious adverse reactions and follows up on these patients 1 year later.

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The potential importance of steroids in the treatment of autistic spectrum disorders and other disorders involving mercury toxicity

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Summary Autism is a neurodevelopmental disorder that according to the Centers for Disease Control and Prevention (CDC) affects 1 in 150 children in the United States. Autism is characterized by impairments in social relatedness and communication, repetitive behaviors, abnormal movements, and sensory dysfunction. Recently emerging evidence suggests that mercury, especially from childhood vaccines, appears to be a factor in the development of the autistic disorders, and that autistic children have higher than normal body-burdens of mercury. In considering mercury toxicity, it has previously been shown that testosterone significantly potentates mercury toxicity, whereas estrogen is protective. Examination of autistic children has shown that the severity of autistic disorders correlates with the amount of testosterone present in the amniotic fluid, and an examination of a case-series of autistic children has shown that some have plasma testosterone levels that were significantly elevated in comparison neurotypical control children. A review of some of the current biomedical therapies for autistics, such as glutathione and cysteine, chelation, secretin, and growth hormone, suggests that they may in fact lower testosterone levels. We put forward the medical hypothesis that autistic disorders, in fact, represents a form of testosterone mercury toxicity, and based upon this observation, one can design novel treatments for autistics directed towards higher testosterone levels in autistic children. We suggest a series of experiments that need to be conducted in order to evaluate the exact mechanisms for mercury–testosterone toxicity, and various types of clinical manipulations that may be employed to control testosterone levels. It is hoped by devising therapies that address the steroid hormone pathways, in addition to the current treatments that successful lower heavy metal body-burdens of mercury, will work synergistically to improve clinical outcomes. In light of the fact that there are a number of other diseases that may have a chronic mercury toxicity component, such as Alzheimer's disease, heart disease, obesity, ALS, asthma, and other various forms of autoimmune disorders, it is imperative that further research should be conducted to understand mercury–testosterone toxicity.

Introduction

Autism is a neurodevelopmental disorder characterized by impairments in social relatedness and

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Thimerosal induced changes of intracellular calcium in human endothelial cells

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Abstract — We have measured the effects of the -SH oxidizing agent thimerosal on the intracellular calcium concentration in single endothelial cells from human umbilical cord vein.

Application of 1 μM thimerosal after a 10 s prepulse of 10 μM evoked oscillations of intracellular calcium. Concentrations higher than 10 μM induced a few oscillations which were followed by a long lasting increase in intracellular calcium between 120 and 980 nM at 10 μM thimerosal, between 250 and 1290 nM at 100 μM.

The plateau level of the thimerosal induced increase in intracellular calcium depended on the extracellular calcium concentration, and was clearly decreased in calcium free solution. It was also reduced if the extracellular potassium concentration was increased to 140 mM. Nickel (5 mM) did not block the elevation of intracellular calcium. Thimerosal induced quenching of the Fura-2 fluorescence in Ca^{2+} free solutions containing 1 mM Mn^{2+}. These effects indicate that thimerosal opens a pathway for Ca^{2+} entry from the extracellular side.

The amount of calcium which could be released by histamine was drastically reduced after initiation of the thimerosal response. If refilling of Ca^{2+} stores was prevented by incubation of the cells in Ca^{2+} free solution, histamine still induced a transient, but not maintained, increase in $[Ca^{2+}]_i$. After application of thimerosal in Ca^{2+} free solutions to prevent refilling of the stores, a transient increase in $[Ca^{2+}]_i$ could still be recorded but the histamine response on $[Ca^{2+}]_i$ almost disappeared indicating a discharge of Ca^{2+} stores by thimerosal.

It is concluded that thimerosal induces long lasting elevations of the intracellular calcium concentration by emptying intracellular agonist sensitive Ca^{2+} pools and activating a transmembrane Ca^{2+} entry from the extracellular space.

Many physiological functions in non-excitable cells are modulated by changes in intracellular calcium ($[Ca^{2+}]_i$). Compounds which disturb intracellular Ca^{2+} homeostasis are often connected to oxidation of groups. It is well known that the oxidizing compound thimerosal induces long-lasting vasodilation. It inhibits the enzyme acyl-coenzyme A : lysolecithin-acyltransferase [1]. As a consequence,

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REPETITIVE TRANSIENTS IN INTRACELLULAR Ca^{2+} IN CULTURED HUMAN VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY

Human uterine vascular smooth muscle cells have been isolated and maintained in culture. When these cells are exposed to bathing solutions with nominally zero sodium, using potassium, *N*-methyl-D-glucamine or Tris as substitutes, repetitive transient increases in intracellular calcium are observed. These transients are abolished when the calcium concentration of the bathing solution is reduced to nominally zero suggesting a role for extracellular calcium in the activation or maintenance of the transients. The hypothesis is proposed that the underlying mechanism involves a calcium influx through the reversed operation of a sodium–calcium exchange mechanism and the cyclical activation of calcium-induced calcium release from the sarcoplasmic reticulum. Noradrenaline (10^{-6} M) and caffeine (20–30 mM) reversibly inhibited the transients. The inhibitory action of these agents could not be mimicked by dibutyryl cAMP suggesting that cAMP does not mediate the inhibition. Caffeine alone had no effect on resting calcium. Thimerosal (1–100 μM), an agent thought to activate a second type of calcium-induced calcium release mechanism activated repetitive transient increases in intracellular calcium which behave in a similar manner to those activated by sodium removal. These data are consistent with the presence of a thimerosal-activated calcium-induced calcium release mechanism in these cultured human cells. It is proposed that this mechanism is different from the calcium-induced calcium release mechanism, described in other cell types, which is activated by caffeine.

INTRODUCTION

The contractile state of vascular smooth muscle is regulated by the concentration of intracellular free calcium ($[Ca^{2+}]_i$). The mechanisms by which Ca^{2+} is elevated can involve either the entry of extracellular Ca^{2+} through voltage- or ligand-gated ion channels or by the release of Ca^{2+} from intracellular stores. It is now evident that intracellular Ca^{2+} is stored in at least two different releasable pools, defined by the primary mechanism which brings about its release. In some cell types it has been demonstrated that there is an inositol trisphosphate (IP_3)-sensitive pool while in other cells there may be a second pool, activated by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Berridge, Cobbold & Cuthbertson, 1988). The functional significance of these different pools in vascular smooth muscle is not clear.

In many cell types in which a CICR mechanism has been identified, it has been shown to be sensitive to methylxanthines. For example in cardiac and skeletal muscle, caffeine is known to elevate $[Ca^{2+}]_i$ by increasing the permeability of the sarcoplasmic reticulum to

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Comparison of VAERS fetal-loss reports during three consecutive influenza seasons: Was there a synergistic fetal toxicity associated with the two-vaccine 2009/2010 season?

GS Goldman

Abstract

The aim of this study was to compare the number of inactivated-influenza vaccine–related spontaneous abortion and stillbirth (SB) reports in the Vaccine Adverse Event Reporting System (VAERS) database during three consecutive flu seasons beginning 2008/2009 and assess the relative fetal death reports associated with the two-vaccine 2009/2010 season. The VAERS database was searched for reports of fetal demise following administration of the influenza vaccine/vaccines to pregnant women. Utilization of an independent surveillance survey and VAERS, two-source capture–recapture analysis estimated the reporting completeness in the 2009/2010 flu season. Capture–recapture demonstrated that the VAERS database captured about 13.2% of the total 1321 (95% confidence interval (CI): 815–2795) estimated reports, yielding an ascertainment-corrected rate of 590 fetal-loss reports per million pregnant women vaccinated (or 1 per 1695). The unadjusted fetal-loss report rates for the three consecutive influenza seasons beginning 2008/2009 were 6.8 (95% CI: 0.1–13.1), 77.8 (95% CI: 66.3–89.4), and 12.6 (95% CI: 7.2–18.0) cases per million pregnant women vaccinated, respectively. The observed reporting bias was too low to explain the magnitude increase in fetal-demise reporting rates in the VAERS database relative to the reported annual trends. Thus, a synergistic fetal toxicity likely resulted from the administration of both the pandemic (A-H1N1) and seasonal influenza vaccines during the 2009/2010 season.

Keywords

Human toxicology, immunization, influenza vaccine, spontaneous abortion, stillbirth, Thimerosal

Introduction

Since 1997, the Advisory Committee on Immunization Practices (ACIP) has recommended the routine vaccination of pregnant women with trivalent inactivated influenza vaccine (TIV) after the first trimester of pregnancy. This recommendation was expanded in 2004 to include all trimesters of pregnancy.[1]

All previously published studies of pregnant women who were administered with TIV have reported this vaccine as safe during all stages of pregnancy.[2–4] Christian et al. explained the reason for this record of safety: 'The inflammatory response elicited by TIV is substantially milder and more transient than seen in infectious illness.'[5]

Two frequently cited peer-reviewed reports on the safety of influenza vaccination during pregnancy did not reveal any adverse outcomes among 56 women[6] and 180 women.[7] Both these studies, which used 'no Thimerosal' influenza vaccines, had insufficient statistical power to adequately detect and assess complications due to the small sample size. A third follow-up safety study (conducted among 2291

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Increased expression of procoagulant activity on the surface of human platelets exposed to heavy-metal compounds

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One of the essential roles for platelets in haemostasis is in the potentiation of blood clotting due to the contribution of anionic phospholipid from the surface of the cells, as an essential cofactor to the proteolytic reactions of coagulation (platelet procoagulant activity). Only a limited number of agonists are known to initiate platelet procoagulant activity. In this study the rate of thrombin formation on the platelet surface was observed to increase in a dose-dependent manner upon treatment of washed platelets with heavy-metal compounds. Unlike the immediate increase observed upon treatment of platelets with calcium ionophore, A23187, the change due to these agents was progressive, approaching a maximum after 10 min. The maximum-fold acceleration of the rate of thrombin formation compared with control platelets was calculated for $HgCl_2$ (56-fold), $AgNO_3$ (42-fold) phenylmercuri-acetate (24-fold) and thimerosal (14-fold), compared with 70-fold observed for calcium ionophore. The increase in procoagulant activity due to $HgCl_2$ coincided with a large increase in intracellular calcium and phosphorylation of 22 and 45 kDa proteins. It is considered that the mechanism responsible for the increase in procoagulant activity is exposure of anionic phospholipids. This was detected by a 2-fold increase in the binding of ^{125}I-annexin V upon addition of $HgCl_2$, compared with resting platelets (3-fold on treatment of platelets with calcium ionophore). In contrast to the generation of activity by A23187 and other known agonists of this reaction, heavy-metal compounds appeared to cause little or no release of microparticles from the platelet surface. Since $HgCl_2$ did not cause aggregation of platelets or significant release of serotinin, these findings may give further support to the need for exposure and ligation of glycoprotein IIb:IIIa for vesiculization to occur. Treatment of platelets with heavy metals may constitute a new approach to investigating the early changes in the cell membrane which lead to increased expression of anionic phospholipid.

INTRODUCTION

Platelets play two important roles in normal haemostasis. First, by aggregating, they constitute the initial haemostatic plug which immediately curtails bleeding from broken blood vessels. Secondly, the platelet surface can become activated and potentiate blood clotting, a property referred to as platelet procoagulant activity. This is usually observed as an increase in the rate of activation of prothrombin by factor Xa in the presence of factor Va and Ca^{2+}, referred to as the prothrombinase reaction. The change to the surface of the platelet responsible for procoagulant activity is due, principally, to a reversal of the polarity of the phospholipid membrane: the anionic phospholipids, which are normally maintained by a translocase system at a higher concentration on the inner leaflet, become exposed on the outer membrane surface (reviewed in [1,2,3,4]). The physiological importance of this property of platelets is demonstrated by the moderately severe bleeding disorder, Scott syndrome, in which stimulated platelets reveal abnormally low levels of anionic phospholipid exposure and a correspondingly lower procoagulant activity [5,6].

The generation of platelet procoagulant activity does not occur with all agonists. 'Weak' agonists such as ADP, adrenalin and platelet-activating factor hardly affect the procoagulant properties of the platelet surface even though irreversible thromboxane-dependent aggregation can proceed to near completion. In contrast, thrombin, collagen, complement attack complex C5b-9 and calcium ionophore have been demonstrated to enhance the generation of platelet procoagulant activity in the order of potency: ionophore > collagen/thrombin > C5b-9 > collagen > thrombin [4]. Treatment of platelets with local anaesthetics (dibucaine and tetracaine) or with sulphydryl oxidizing agents (diamide or pyridyldithiolethanolamine) also cause an increase in procoagulant activity which is dependent upon extracellular calcium [4].

We recently observed an increase in the procoagulant activity of U937 monocyte-like cells upon treatment with mercuric and other heavy-metal compounds [7]. Both the tissue factor activity of the cell and the ability of the surface to support the prothrombinase reaction were rapidly increased, concomitant with a large increase in intracellular calcium concentration ($[Ca^{2+}]_i$). In the present study we have identified these heavy metals as potent agonists of platelet procoagulant activity. The characteristics of induction of the procoagulant surface are distinct from that promoted by other platelet agonists in that the degree of microvesiculization is low.

EXPERIMENTAL

Materials

Human α-thrombin was obtained as a gift from Dr. J.-M. Freysinnet (Strasbourg, France), human factor X from Enzyme Research Laboratories (Swansea, U.K.) and bovine factor V

Abbreviations used: PRP, platelet-rich plasma; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular calcium concentration; [^{14}C]5-HT, [^{14}C]5-hydroxytryptamine; MLC, myosin light chain; $[Ca^{2+}]_{cyt.}$, cytosolic calcium; Tg, thapsigargin.

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MC1568 Inhibits Thimerosal-Induced Apoptotic Cell Death by Preventing HDAC4 Up-Regulation in Neuronal Cells and in Rat Prefrontal Cortex

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ABSTRACT

Ethylmercury thiosalicylate (thimerosal) is an organic mercury-based compound commonly used as an antimicrobial preservative that has been found to be neurotoxic. In contrast, histone deacetylases (HDACs) inhibition has been found to be neuroprotective against several environmental contaminants, such as polychlorinated biphenyls, di-2-ethylhexyl phthalate, and methylmercury. The aim of this study was to investigate the effect of HDAC inhibition on thimerosal-induced neurotoxicity in neuroblastoma cells and cortical neurons. Interestingly, we found that thimerosal, at 0.5 μM in SH-SY5Y cells and at 1 μM in neurons, caused cell death by activation of apoptosis, which was prevented by the HDAC class IIA inhibitor MC1568 but not the class I inhibitor MS275. Furthermore, thimerosal specifically increased HDAC4 protein expression but not that of HDACs 5, 6, 7, and 9. Western blot analysis revealed that MC1568 prevented thimerosal-induced HDAC4 increase. In addition, both HDAC4 knocking-down and MC1568 inhibited thimerosal-induced cell death in SH-SY5Y cells and cortical neurons. Importantly, intramuscular injection of 12 μg/kg thimerosal on postnatal days 7, 9, 11, and 15 increased HDAC4 levels in the prefrontal cortex (PFC), which decreased histone H4 acetylation in infant male rats, in parallel increased motor activity changes. In addition, coadministration of 40 mg/kg MC1568 (intraperitoneal injection) moderated the HDAC4 increase which reduced histone H4 deacetylation and caspase-3 cleavage in the PFC. Finally, open-field testing showed that thimerosal-induced motor activity changes are reduced by MC1568. These findings indicate that HDAC4 regulates thimerosal-induced cell death in neurons and that treatment with MC1568 prevents thimerosal-induced activation of caspase-3 in the rat PFC.

Key words: HDAC; MC1568; thimerosal.

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INORGANIC COMPOUNDS

Ethylmercury and Hg^{2+} induce the formation of neutrophil extracellular traps (NETs) by human neutrophil granulocytes

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Abstract Humans are exposed to different mercurial compounds from various sources, most frequently from dental fillings, preservatives in vaccines, or consumption of fish. Among other toxic effects, these substances interact with the immune system. In high doses, mercurials are immunosuppressive. However, lower doses of some mercurials stimulate the immune system, inducing different forms of autoimmunity, autoantibodies, and glomerulonephritis in rodents. Furthermore, some studies suggest a connection between mercury exposure and the occurrence of autoantibodies against nuclear components and granulocyte cytoplasmic proteins in humans. Still, the underlying mechanisms need to be clarified. The present study investigates the formation of neutrophil extracellular traps (NETs) in response to thimerosal and its metabolites ethyl mercury (EtHg), thiosalicylic acid, and mercuric ions (Hg^{2+}). Only EtHg and Hg^{2+} triggered NETosis. It was independent of PKC, ERK1/2, p38, and zinc signals and not affected by the NADPH oxidase inhibitor DPI. Instead, EtHg and Hg^{2+} triggered NADPH oxidase-independent production of ROS, which are likely to be involved in mercurial-induced NET formation. This finding might help understanding the autoimmune potential of mercurial compounds. Some diseases, to which a connection with mercurials has been shown, such as Wegener's granulomatosis and systemic lupus erythematosus, are characterized by high prevalence of autoantibodies against neutrophil-specific auto-antigens. Externalization in the form of NETs may be a source for exposure to these self-antigens. In genetically susceptible individuals, this could be one step in the series of events leading to autoimmunity.

Keywords Neutrophil extracellular traps · PMN · Granulocytes · Autoimmunity · Mercurials · Ethylmercury

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Introduction

Human exposure to mercury results from several different sources, including inorganic mercury from amalgam-based tooth fillings and organic compounds, such as methyl mercury (MeHg) from fish or ethyl mercury (EtHg), which can be released from preservatives in vaccines (Clarkson et al. 2007). EtHg is a metabolite of thimerosal (TMS), which readily dissociates into thiosalicylic acid (TSA) and EtHg (Elferink 1999). Organic mercurial compounds can also be converted into inorganic mercuric ions (Hg^{2+}), and several days after treatment of mice with TMS, a significant portion is found in the form of inorganic mercury in the kidneys (Havarinasab et al. 2005). Exposure to high doses of mercury is characterized predominantly by neurotoxicity and nephrotoxicity. Although immune cells are also affected by mercury toxicity, this is usually eclipsed by the severe and life-threatening neurotoxic effects (Vas and Monestier 2008). Nonetheless, immunotoxicity can already be observed at much lower concentrations at which, in general, mercurial compounds are considered to be immunosuppressive (Havarinasab and Hultman 2005).

Neutrophil granulocytes are the major cell population of the innate immune system. During infection, they migrate in high numbers toward the infected site to take up

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Increase in intracellular Zn^{2+} concentration by thimerosal in rat thymocytes: Intracellular Zn^{2+} release induced by oxidative stress

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ABSTRACT

Thimerosal (TMR), an ethylmercury-containing preservative in pharmaceutical products, was recently reported to increase intracellular Zn^{2+} concentration. Therefore, some health concerns about the toxicity of TMR remain because of physiological and pathological roles of Zn^{2+}. To reveal the property of TMR-induced increase in intracellular Zn^{2+} concentration, the effect of TMR on FluoZin-3 fluorescence, an indicator of intracellular Zn^{2+}, of rat thymocytes was examined. TMR at concentrations ranging from 0.3 μM to 10 μM increased the intensity of FluoZin-3 fluorescence in a concentration-dependent manner under external Ca^{2+}- and Zn^{2+}-free condition. The threshold concentration was 0.3–1 μM. The increase in the intensity was significant when TMR concentration was 1 μM or more. *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a chelator for intracellular Zn^{2+}, completely attenuated the TMR-induced augmentation of FluoZin-3 fluorescence. Hydrogen peroxide (H_2O_2) and *N*-ethylmaleimide, reducing cellular thiol content, significantly increased FluoZin-3 fluorescence intensity and decreased 5-chloromethylfluorescein (5-CMF) fluorescence intensity, an indicator for cellular thiol. The correlation coefficient between TMR-induced augmentation of FluoZin-3 fluorescence and attenuation of 5-CMF fluorescence was −0.882. TMR also attenuated the 5-CMF fluorescence in the presence of TPEN. Simultaneous application of H_2O_2 and TMR synergistically augmented the FluoZin-3 fluorescence. It is suggested that TMR increases intracellular Zn^{2+} concentration *via* decreasing cellular thiol content.

1. Introduction

Thimerosal (TMR) is an ethylmercury-containing preservative used in vaccines, immune globulin preparations, and other pharmaceutical products. Because it contains 49.55% mercury, it has been hypothesized that early exposure to this preservative is associated with neuropsychological deficits in children (Bernard et al., 2001; Redwood et al., 2001). Although there are many clinical and basic studies against the hypothesis (Ball et al., 2001; Magos, 2003; Verstraeten et al., 2003; Andrews et al., 2004; Burbacher et al., 2005; Zareba et al., 2007; Thompson et al., 2007), some health concerns about the toxicity of TMR remain (Geier et al., 2007; Berman et al., 2008).

The cytotoxicity of TMR has been discussed in the phenomena related to the disturbance of intracellular Ca^{2+} homeostasis. TMR increases intracellular Ca^{2+} concentration by increasing membrane Ca^{2+} permeability and releasing Ca^{2+} from intracellular calcium stores *via* inhibition of Ca^{2+} pump or sensitization of IP_3 receptor at endoplasmic reticulum membranes (Gukovskaya et al., 1992; Thorn et al., 1992; Sayers et al., 1993; Parys et al., 1993). In addition, it has been recently proposed that TMR increases intracellular Zn^{2+} concentration *via* an intracellular Zn^{2+} release (Haase et al., 2009). Zn^{2+} is the second most prevalent trace element and it is involved in the structure and function of over 300 enzymes (Prasad, 1995). Zn^{2+} stimulates the activity of approximately 100 enzymes (Sandstead, 1994). Therefore, an abnormal increase in intracellular Zn^{2+} concentration may cause pathological phenomena.

Intracellular Zn^{2+} is complexed to thiol of protein and nonprotein such as metallothionein and glutathione (Diaz-Cruz et al., 1998; Jacob et al., 1998; Maret and Vallee, 1998; Gelinsky et al., 2003). Oxidative stress releases Zn^{2+} from protein and nonprotein *via* interchange between thiol and disulfide (Maret, 1994; Quesada et al., 1996). TMR induces oxidative stress (Makani et al., 2002; Ueha-Ishibashi et al., 2004; James et al., 2005). Therefore, it is reminiscent of a possibility that TMR increases intracellular Zn^{2+}

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Dose–response study of thimerosal-induced murine systemic autoimmunity

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Abstract

The organic compound ethylmercurithiosalicylate (thimerosal), which is primarily present in the tissues as ethylmercury, has caused illness and several deaths due to erroneous handling when used as a disinfectant or as a preservative in medical preparations. Lately, possible health effects of thimerosal in childhood vaccines have been much discussed. Thimerosal is a well-known sensitizing agent, although usually of no clinical relevance. In rare cases, thimerosal has caused systemic immune reactions including acrodynia. We have studied if thimerosal might induce the systemic autoimmune condition observed in genetically susceptible mice after exposure to inorganic mercury.

A.SW mice were exposed to 1.25–40 mg thimerosal/l drinking water for 70 days. Antinucleolar antibodies, targeting the 34-kDa protein fibrillarin, developed in a dose-related pattern and first appeared after 10 days in the two highest dose groups. The lowest observed adverse effect level (LOAEL) for antifibrillarin antibodies was 2.5 mg thimerosal/l, corresponding to an absorbed dose of 147 μg Hg/kg bw and a concentration of 21 and 1.9 μg Hg/g in the kidney and lymph nodes, respectively. The same LOAEL was found for tissue immune-complex deposits. The total serum concentration of IgE, IgG1, and IgG2a showed a significant dose-related increase in thimerosal-treated mice, with a LOAEL of 5 mg thimerosal/l for IgG1 and IgE, and 20 mg thimerosal/l for IgG2a. The polyclonal B-cell activation showed a significant dose–response relationship with a LOAEL of 10 mg thimerosal/l. Therefore, thimerosal induces in genetically susceptible mice a systemic autoimmune syndrome very similar to that seen after treatment with inorganic mercury, although a higher absorbed dose of Hg is needed using thimerosal. The autoimmune syndrome induced by thimerosal is different from the weaker and more restricted autoimmune reaction observed after treatment with an equipotent dose of methylmercury.

Keywords: Thimerosal; Ethylmercury; Mice; Autoimmunity

Introduction

In a recent review Clarkson (2002) described thimerosal in vaccines as one of three modern faces of mercury, the two other being methylmercury in fish and mercury vapor from dental amalgam fillings. Thimerosal is an organic, alkylmercury compound in which an organic radical, ethylmercury, is bound to the sulfur atom of the thiol group of salicylic acid. The type of anion attached to ethylmercury affects neither the distribution of mercury in the body nor the toxicity (Suzuki et al., 1973; Ulfvarson, 1962), while the organic radical has a strong impact on both (Magos, 2003). Ethylmercury and its decomposition product, Hg^{2+}, rapidly accumulate in the tissues (Magos, 2001).

Ethylmercury has been frequently used since it was first synthesized in the 19th century. When used as a seed disinfectant in developing countries, it caused several outbreaks of poisoning with neurological symptoms and signs similar to those of methylmercury intoxication (Clarkson, 2002). Such manifestations have also been recorded after occupational exposure and after use as a wound disinfectant and a preservative in medical preparations (Magos, 2001). A number of severe intoxications and deaths have occurred with the use of erroneous concentrations of thimerosal in medical preparations during the last 30 years (Axton, 1972; Suzuki et al., 1973).

Since the 1930s, thimerosal has been used world-wide as a preservative in vaccines, a use that was approved as late as 1976 by the U.S. Food and Drug Administration

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Immunosuppressive and autoimmune effects of thimerosal in mice

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Abstract

The possible health effects of the organic mercury compound thimerosal (ethylmercurithiosalicylate), which is rapidly metabolized to ethylmercury (EtHg), have recently been much debated and the effect of this compound on the immune system is largely unknown. We therefore studied the effect of thimerosal by treating A.SW (H-2^s) mice, susceptible to induction of autoimmunity by heavy metals, with 10 mg thimerosal/L drinking water (internal dose ca 590 μg Hg/kg body weight/day) for up to 30 days. The lymph node expression of IL-2 and IL-15 mRNA was increased after 2 days, and of IL-4 and IFN-γ mRNA after 6 and 14 days. During the first 14 days treatment, the number of splenocytes, including T and B cells as well as Ig-secreting cells decreased. A strong immunostimulation superseded after 30 days treatment with increase in splenic weight, number of splenocytes including T and B cells and Ig-secreting cells, and Th2- as well as Th-1-dependent serum immunoglobulins. Antinucleolar antibodies (ANoA) targeting the 34-kDa nucleolar protein fibrillarin, and systemic immune-complex deposits developed. The H-2^s strains SJL and B10.S also responded to thimerosal treatment with ANoA. The A.TL and B10.TL strain, sharing background genes with the A.SW and B10.S strain, respectively, but with a different H-2 haplotype (*t1*), did not develop ANoA, linking the susceptibility to H-2. Thimerosal-treated H-2^s mice homozygous for the *nu* mutation (SJL-*nu/nu*), or lacking the T-cell co-stimulatory molecule CD28 (B10.S–CD28$^{-/-}$), did not develop ANoA, which showed that the autoimmune response is T-cell dependent. Using H-2^s strains with targeted mutations, we found that IFN-γ and IL-6, but not IL-4, is important for induction of ANoA by thimerosal. The maximum added renal concentration of thimerosal (EtHg) and inorganic mercury occurred after 14 days treatment and was 81 μg Hg/g. EtHg made up 59% and inorganic mercury 41% of the renal mercury. In conclusion, the organic mercury compound thimerosal (EtHg) has initial immunosuppressive effects similar to those of MeHg. However, in contrast to MeHg, thimerosal treatment leads in genetically susceptible mice to a second phase with strong immunostimulation and autoimmunity, which is T-cell dependent, H-2 linked and may at least partly be due to the inorganic mercury derived from the metabolism of ethyl mercury.

Keywords: Thimerosal; Ethylmercury; Mice; Immunosuppression; Autoimmunity

Introduction

Thimerosal has for a long time been used as a wound disinfectant and a preservative in medical preparations, not least human vaccines (Magos, 2001). However, more extensive childhood immunization schedules and increased concern regarding the potential effect of low level exposure of organic mercurials on neurodevelopment, recently raised the question of thimerosal in vaccines as a public health concern (Stratton et al., 2001a). As a precautionary measure, the use of thimerosal in vaccines has now been largely abandoned in the US (Ball et al., 2001).

Knowledge on the toxicokinetics and toxicology of thimerosal is limited (Clarkson, 2002), and to a large extent based on comparisons with methyl mercury (MeHg), which due to its presence as a common environmental contaminant has been more intensely studied (Stratton et al., 2001b). Thimerosal consists of an organic radical, ethylmercury (EtHg), bound to the sulfur atom of the thiol group of

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Organic mercury compounds and autoimmunity☆

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Abstract

Based on in vitro studies and short-term in vivo studies, all mercurials were for a long time considered as prototypic immunosuppressive substances. Recent studies have confirmed that organic mercurials such as methyl mercury (MeHg) and ethyl mercury (EtHg) are much more potent immunosuppressors than inorganic mercury (Hg). However, Hg interacts with the immune system in the presence of a susceptible genotype to cause immunostimulation, antinucleolar antibodies targeting fibrillarin, and systemic immune-complex (IC) deposits, a syndrome called Hg-induced autoimmunity (HgIA). Recent studies in mice with a susceptible genotype has revealed that the immunosuppressive effect of MeHg and EtHg will within 1–3 weeks be superseded by immunostimulation causing an HgIA-like syndrome. At equimolar doses of Hg, MeHg has the weakest immunostimulating, autoimmunogen, and IC-inducing effect, while the effect of thimerosal is similar to that of inorganic mercury. The immunosuppression is caused by the organic mercurials per se. Since they undergo rapid transformation to inorganic Hg, studies are being undertaken to delineate the importance of the organic substances per se and the newly formed inorganic Hg for induction of autoimmunity.

Keywords: Methyl mercury; Thimerosal; Mice; Immunosuppression; Autoimmunity

Contents

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Alteration of the spontaneous systemic autoimmune disease in (NZB × NZW)F1 mice by treatment with thimerosal (ethyl mercury)

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Abstract

Inorganic mercury may aggravate murine systemic autoimmune diseases which are either spontaneous (genetically determined) or induced by non-genetic mechanisms. Organic mercury species, the dominating form of mercury exposure in the human population, have not been examined in this respect. Therefore, ethyl mercury in the form of thimerosal, a preservative recently debated as a possible health hazard when present in vaccines, was administered in a dose of 0.156–5 mg/L drinking water to female (NZB × NZW)F1 (ZBWF1) mice. These mice develop an age-dependent spontaneous systemic autoimmune disease with high mortality primarily due to immune-complex (IC) glomerulonephritis. Five mg thimerosal/L drinking water (295 μg Hg/kg body weight (bw)/day) for 7 weeks induced glomerular, mesangial and systemic vessel wall IC deposits and antinuclear antibodies (ANA) which were not present in the untreated controls. After 22–25 weeks, the higher doses of thimerosal had shifted the localization of the spontaneously developing renal glomerular IC deposits from the capillary wall position seen in controls to the mesangium. The altered localization was associated with less severe histological kidney damage, less proteinuria, and reduced mortality. The effect was dose-dependent, lower doses having no effect compared with the untreated controls. A different effect of thimerosal treatment was induction of renal and splenic vessel walls IC deposits. Renal vessel wall deposits occurred at a dose of 0.313–5 mg thimerosal/L (18–295 μg Hg/kg bw/day), while splenic vessel wall deposits developed also in mice given the lowest dose of thimerosal, 0.156 mg/L (9 μg Hg/kg bw/day). The latter dose is 3- and 15-fold lower than the dose of Hg required to induce vessel wall IC deposits in genetically susceptible H-2^s mice by $HgCl_2$ and thimerosal, respectively. Further studies on the exact conditions needed for induction of systemic IC deposits by low-dose organic mercurials in autoimmune-prone individuals, as well as the potential effect of these deposits on the vessel walls, are warranted.

Keywords: Thimerosal; Mice; Autoimmunity; Immune-complex; (NZB × NZW)F1 mice

Introduction

Thimerosal (ethylmercurithiosalicylate) has for a long time been used in medical preparations, not least human vaccines (Magos, 2001). However, more extensive childhood immunization schedules recently raised the question of thimerosal in vaccines as a possible public health issue due to concern for neurodevelopmental effects, especially autistic spectrum disorders (ASD) (Stratton et al., 2001). Although recent reviews did not find a link between thimerosal-containing vaccines and ASD (IOM, 2004; Parker et al., 2004), the use of thimerosal in vaccines has now been largely abandoned in the US (Ball et al., 2001). However, thimerosal-containing vaccines are recommended by the WHO for use in developing countries due to their cost effectiveness and logistical suitability (Bigham and Copes, 2005), which means that the number of individuals globally exposed to thimerosal will continue to be large. Knowledge on the toxicology of thimerosal is limited (Clarkson, 2002) and based mainly on comparison with methyl mercury (MeHg) (Magos, 2001). However, the toxicokinetics of ethyl mercury (EtHg), the active component of thimerosal, may differ substantially from that of MeHg (Harry et al., 2004). Further studies on the toxicology

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THE SULFHYDRYL REAGENT THIMEROSAL ELICITS HUMAN PLATELET AGGREGATION BY MOBILIZATION OF INTRACELLULAR CALCIUM AND SECONDARY PROSTAGLANDIN ENDOPEROXIDE FORMATION

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Summary. The effect of the sulfhydryl (SH) group inhibitor ethylmercurithiosalicylate (thimerosal) on the function of human platelets was investigated. In contrast to known SH reagents such as *p*-chloromercuribenzoate or N-ethylmaleimide, thimerosal elicited both aggregation and [^{3}H]serotonin release of washed human platelets at low micromolar concentrations ($\geq$ 2 μM). Only a significant higher dose ($\geq$ 15 μM) was effective when platelets were pretreated with the cyclooxygenase inhibitor aspirin, indicating an amplification of the proaggregatory effect of thimerosal by secondary prostaglandin (PG) endoperoxide and/or thromboxane (TX) formation. Consistent with this notion, thimerosal induced endogenous platelet arachidonic acid (20:4) metabolism which could be attributed to enhanced 20:4 liberation, presumably by activation of phospholipase A_2. The latter effect was mediated by mobilization of intracellular calcium (Ca^{2+}), and was not affected by removal of extracellular Ca^{2+}. In the presence of aspirin, the thimerosal-induced Ca^{2+} elevation was completely reversed by dithiothreitol (DTT) which implicates SH groups in intracellular Ca^{2+} transport. In contrast to previous observations with other SH reagents, thimerosal had no effect on the inositoltrisphosphate (IP_3)-mediated release or the sequestration (and/or extrusion) of intracellular Ca^{2+} following stimulation with thrombin, indicating an action on an as yet undefined Ca^{2+} transport system.

Platelet membranes contain SH groups essential for the maintenance of platelet integrity and function, and SH reagents affect platelet function by binding to SH and disulfide groups of platelet membranes [1]. The organic mercury compound thimerosal elicits aggregation of platelet-rich plasma and serotonin release, presumably by such a mechanism [2,3]. Moreover, thimerosal induces release of the endothelium-derived relaxing factor (EDRF) from endothelial cells, probably a Ca^{2+}-mediated process [4,5], and stimulates arachidonic acid (20:4) metabolism in human platelets and murine peritoneal macrophages [6]. The latter effect has been attributed to inhibition of 20:4 reacylation leading to an increased level of free 20:4 within the cell [7], generally accepted to be the limiting factor in eicosanoid biosynthesis [8]. On the contrary, esterified 20:4 can be liberated from cellular (phospho)lipids by phospholipase A_2 in response to an elevation of the intracellular Ca^{2+} level by various agonists [9]. Platelets convert 20:4 mainly to the PG endoperoxide PGH_2 which is subsequently metabolized to 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and TXA_2 by TX synthase [10]. PGH_2 and in particular TXA_2 are powerfull platelet agonists which stimulate phosphatidylinositol (PI) metabolism [11]. The present study addresses the question by which mechanism thimerosal increases the level of free 20:4 in platelets and whether this effect accounts for its proaggregatory activity.

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Thimerosal Induces Apoptosis in a Neuroblastoma Model via the cJun N-Terminal Kinase Pathway

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The cJun N-terminal kinase (JNK)-signaling pathway is activated in response to a variety of stimuli, including environmental insults, and has been implicated in neuronal apoptosis. In this study, we investigated the role that the JNK pathway plays in neurotoxicity caused by thimerosal, an ethylmercury-containing preservative. SK-N-SH cells treated with thimerosal (0–10μM) showed an increase in the phosphorylated (active) form of JNK and cJun with 5 and 10μM thimerosal treatment at 2 and 4 h. To examine activator protein-1 (AP-1) transcription, cells were transfected with a pGL2 vector containing four AP-1 consensus sequences and then treated with thimerosal (0–2.5μM) for 24 h. Luciferase studies showed an increase in AP-1 transcriptional activity upon thimerosal administration. To determine the components of the AP-1 complex, cells were transfected with a dominant negative to either cFos (A-Fos) or cJun (TAM67). Reporter analysis showed that TAM67, but not A-Fos, decreased AP-1 transcriptional activity, indicating a role for cJun in this pathway. To assess which components are essential to apoptosis, cells were treated with a cell-permeable JNK inhibitor II (SP600125) or transfected with TAM67, and the downstream effectors of apoptosis were analyzed. Cells pretreated with SP600125 showed decreases in activation of caspases 9 and 3, decreases in degradation of poly(ADP-ribose) polymerase (PARP), and decreased levels of proapoptotic Bim, in comparison to cells treated with thimerosal alone. However, cells transfected with TAM67 showed no changes in those same components. Taken together, these results indicate that thimerosal-induced neurotoxicity occurs through the JNK-signaling pathway, independent of cJun activation, leading ultimately to apoptotic cell death.

Key Words: **thimerosal; mercury; neurotoxicity; JNK; cJun; Bim.**

Thimerosal is an organic mercurial containing an ethylmercury moiety attached to the sulfur atom of thiosalicylate. Since the 1930's, thimerosal has been used in many products as an antiseptic and a preservative. In recent years, controversy has surrounded the use of thimerosal in vaccines as mercury is a known neurotoxin and nephrotoxin. Since the controversy began in the late 1990's, much of the thimerosal has been removed from vaccines administered to children in the United States. However, it remains in some, such as the influenza vaccine, and is added to multidose vials used in countries around the world. Studies concentrating on thimerosal-induced neurotoxicity are limited, and exposure guidelines, such as those set by the Food and Drug Administration, are based on research with methylmercury. Interestingly, some *in vitro* and *in vivo* studies suggest that ethylmercury may react differently than methylmercury (Aschner and Aschner, 1990; Harry *et al.*, 2004; Magos *et al.*, 1985). Few studies with thimerosal have focused on determining specific signaling pathways involved in neurotoxicity. Establishing these pathways may be an important step in discovering methods of alleviating toxic outcomes in patients exposed to thimerosal.

While the toxicological profile of thimerosal is still somewhat limited, the amount of information regarding thimerosal-induced toxicity is increasing. Recent studies have shown various events occurring in response to thimerosal exposure. Rat cerebellar neurons treated with thimerosal showed increases in intracellular calcium levels and decreases in glutathione levels (Ueha-Ishibashi *et al.*, 2004a, 2005). Decreased glutathione levels resulting from thimerosal exposure were also seen in rat thymocytes (Ueha-Ishibashi *et al.*, 2004b), cultured neuroblastoma cells (SH-SY5Y), and glioblastoma cells (James *et al.*, 2005). HeLa S cells treated with thimerosal showed cytoskeletal changes and activation of focal adhesion kinase, both of which were attributed to the production of reactive oxygen species (Kim *et al.*, 2002). Studies in our laboratory have begun to establish a more coordinated picture of events that occur in cells treated with thimerosal. We have shown that upon treatment with thimerosal, SK-N-SH neuroblastoma cells exhibited a time- and concentration-dependent increase in apoptotic cell death, as evidenced by increases in nuclear condensation, cytochrome *c* release, caspases 9 and 3 activation, poly(ADP-ribose) polymerase (PARP) degradation, and lactate dehydrogenase release, thus demonstrating a

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DELAYED ACQUISITION OF NEONATAL REFLEXES IN NEWBORN PRIMATES RECEIVING A THIMEROSAL-CONTAINING HEPATITIS B VACCINE: INFLUENCE OF GESTATIONAL AGE AND BIRTH WEIGHT

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This study examined whether acquisition of neonatal reflexes in newborn rhesus macaques was influenced by receipt of a single neonatal dose of hepatitis B vaccine containing the preservative thimerosal (Th). Hepatitis B vaccine containing a weight-adjusted Th dose was administered to male macaques within 24 h of birth ($n = 13$). Unexposed animals received saline placebo ($n = 4$) or no injection ($n = 3$). Infants were tested daily for acquisition of nine survival, motor, and sensorimotor reflexes. In exposed animals there was a significant delay in the acquisition of *root*, *snout*, and *suck* reflexes, compared with unexposed animals. No neonatal responses were significantly delayed in unexposed animals. Gestational age (GA) and birth weight (BW) were not significantly correlated. Cox regression models were used to evaluate main effects and interactions of exposure with BW and GA as independent predictors and time-invariant covariates. Significant main effects remained for exposure on *root* and *suck* when controlling for GA and BW, such that exposed animals were relatively delayed in time-to-criterion. Interaction models indicated there were various interactions between exposure, GA, and BW and that inclusion of the relevant interaction terms significantly improved model fit. This, in turn, indicated that lower BW and/or lower GA exacerbated the adverse effects following vaccine exposure. This primate model provides a possible means of assessing adverse neurodevelopmental outcomes from neonatal Th-containing hepatitis B vaccine exposure, particularly in infants of lower GA or BW. The mechanisms underlying these effects and the requirements for Th requires further study.

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Influence of pediatric vaccines on amygdala growth and opioid ligand binding in rhesus macaque infants: A pilot study

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This longitudinal, case-control pilot study examined amygdala growth in rhesus macaque infants receiving the complete US childhood vaccine schedule (1994-1999). Longitudinal structural and functional neuroimaging was undertaken to examine central effects of the vaccine regimen on the developing brain. Vaccine-exposed and saline-injected control infants underwent MRI and PET imaging at approximately 4 and 6 months of age, representing two specific timeframes within the vaccination schedule. Volumetric analyses showed that exposed animals did not undergo the maturational changes over time in amygdala volume that was observed in unexposed animals. After controlling for left amygdala volume, the binding of the opioid antagonist [^{11}C]diprenorphine (DPN) in exposed animals remained relatively constant over time, compared with unexposed animals, in which a significant decrease in [^{11}C]DPN binding occurred. These results suggest that maturational changes in amygdala volume and the binding capacity of [^{11}C]DPN in the amygdala was significantly altered in infant macaques receiving the vaccine schedule. The macaque infant is a relevant animal model in which to investigate specific environmental exposures and structural/functional neuroimaging during neurodevelopment.

Key Words: rhesus macaques, *Macaca mulatta*, non-human primates, animal model, neuroimaging, PET, MRI, amygdala, opioids, ethyl mercury, thimerosal, neurotoxicity

INTRODUCTION

The amygdala, a complexly interconnected limbic system structure located in the temporal lobe of the brain, is thought to play a central role in the expression of emotions (reviewed by Aggleton 1992). In rhesus macaques the amygdala has been associated with the development of social and emotional behavior (reviewed by Brothers 1990). When neonatal macaques received lesions to the amygdala they showed increasing socio-emotional disturbances including abnormal social interaction, absence of facial and body expression, and stereotypic behaviors (Bachevalier 1994). Amaral and colleagues reported that infant monkeys with bilateral amygdala lesions were still capable of interpreting and generating social behaviors (Prather et al. 2001) but failed to develop an appropriate fear response (Antoniadis et al. 2009), implicating an important role for the amygdala in regulating such responses (reviewed by Amaral and Corbett 2003, Amaral et al. 2008, Machado et al. 2009, Roozendaal et al. 2009). While the human amygdala has been well studied longitudinally in both normal and disease states, there is a paucity of information regarding amygdala growth during non-human primate development.

Evidence from animal model systems indicates that endogenous opioids play an important role in neural and behavioral ontogeny (Zagon et al. 1982). The primate amygdala has been shown to have a high avidity for opioids. For example, high levels of [^{3}H]diprenorphine (DPN)-binding in the amygdala of healthy adult male cynomolgus monkeys (*Macaca fascicularis*) were

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PROBLEMS ASSOCIATED WITH THE USE OF MERTHIOLATE AS A PRESERVATIVE IN ANTI-LYMPHOCYTIC GLOBULIN

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SUMMARY

The cytotoxic properties of 2 anti-lymphocytic globulin (ALG) preparations were investigated in vitro by measuring the release of ^{51}Cr from labelled human peripheral blood mononuclear cells, tonsil lymphocytes and Chang cells, incubated with different concentrations of ALG. One of the ALG preparations showed non-selective cytotoxicity in the absence of complement. Evidence was obtained to suggest that this effect was due to merthiolate (sodium ethylmercurithiosalicylate) which had been added to the ALG as a preservative during manufacture. The mercury concentration in the ALG was found to be greater than that stated by the manufacturers. It is conceivable that the clinical use of such as ALG preparation might lead to mercury accumulation in the tissues, with resulting toxic effects. The whole question of the use of merthiolate in the preparation of sera for administration to human subjects needs to be reconsidered.

INTRODUCTION

During an investigation of the cytotoxic properties of 2 antilymphocytic globulin (ALG) preparations, carried out in conjunction with a therapeutic trial of ALG in acute ulcerative colitis (Heyworth, M.F. and Truelove, S.C., unpublished), it was found that one of the preparations had a complement-independent cytotoxic effect on several types of target cell. Evidence was obtained to suggest that this effect was the result of merthiolate which had been added to the ALG as a preservative during manufacture.

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Abbreviation: ALG, anti-lymphocytic globulin.

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Review Article

Methodological Issues and Evidence of Malfeasance in Research Purporting to Show Thimerosal in Vaccines Is Safe

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There are over 165 studies that have focused on Thimerosal, an organic-mercury (Hg) based compound, used as a preservative in many childhood vaccines, and found it to be harmful. Of these, 16 were conducted to specifically examine the effects of Thimerosal on human infants or children with reported outcomes of death; acrodynia; poisoning; allergic reaction; malformations; autoimmune reaction; Well's syndrome; developmental delay; and neurodevelopmental disorders, including tics, speech delay, language delay, attention deficit disorder, and autism. In contrast, the United States Centers for Disease Control and Prevention states that Thimerosal is safe and there is "no relationship between [T]himerosal[-]containing vaccines and autism rates in children." This is puzzling because, in a study conducted directly by CDC epidemiologists, a 7.6-fold increased risk of autism from exposure to Thimerosal during infancy was found. The CDC's current stance that Thimerosal is safe and that there is no relationship between Thimerosal and autism is based on six specific published epidemiological studies coauthored and sponsored by the CDC. The purpose of this review is to examine these six publications and analyze possible reasons why their published outcomes are so different from the results of investigations by multiple independent research groups over the past 75+ years.

1. Introduction

Thimerosal is an organic-mercury (Hg) based compound, used as a preservative in many childhood vaccines, in the past and present. To date, there have been over 165 studies that focused on Thimerosal and found it to be harmful [1, 2]. (A comprehensive list of these studies is shown at http://mercury-freedrugs.org/docs/20140329_Kern_JK_ExcelFile_TM_sHarm_ReferenceList_v33.xlsx.) Of these studies, 16 were conducted to specifically examine the effects of Thimerosal on human infants and/or children [3–18]. Within these studies, which focused on human infants and/or children, the reported outcomes following Thimerosal exposure were (1) death [3]; (2) acrodynia [4]; (3) poisoning [5]; (4) allergic reaction [6]; (5) malformations [7]; (6) autoimmune reaction [8]; (7) Well's syndrome [9]; (8) developmental delay [10–13]; and (9) neurodevelopmental disorders, including tics, speech delay, language delay, attention deficit disorder, and autism [10, 11, 14–18].

However, the United States (US) Centers for Disease Control and Prevention (CDC) still insists that there is "no relationship between [T]himerosal[-]containing vaccines and autism rates in children" [19]. This is a puzzling conclusion because, in a study conducted directly by the CDC, epidemiologists assessed the risk for neurologic and renal impairment associated with past exposure to Thimerosal-containing vaccine (TCV) using automated data from the Vaccine Safety Datalink (VSD) and found a 7.6-fold increased risk of autism from exposure to Thimerosal during infancy [20]. The database for that study was "from four health

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NeuroToxicology

Mitochondrial Mediated Thimerosal-Induced Apoptosis in a Human Neuroblastoma Cell Line (SK-N-SH)

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Abstract

Environmental exposure to mercurials continues to be a public health issue due to their deleterious effects on immune, renal and neurological function. Recently the safety of thimerosal, an ethyl mercury-containing preservative used in vaccines, has been questioned due to exposure of infants during immunization. Mercurials have been reported to cause apoptosis in cultured neurons; however, the signaling pathways resulting in cell death have not been well characterized. Therefore, the objective of this study was to identify the mode of cell death in an in vitro model of thimerosal-induced neurotoxicity, and more specifically, to elucidate signaling pathways which might serve as pharmacological targets. Within 2 h of thimerosal exposure (5 μM) to the human neuroblastoma cell line, SK-N-SH, morphological changes, including membrane alterations and cell shrinkage, were observed. Cell viability, assessed by measurement of lactate dehydrogenase (LDH) activity in the medium, as well as the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, showed a time- and concentration-dependent decrease in cell survival upon thimerosal exposure. In cells treated for 24 h with thimerosal, fluorescence microscopy indicated cells undergoing both apoptosis and oncosis/necrosis. To identify the apoptotic pathway associated with thimerosal-mediated cell death, we first evaluated the mitochondrial cascade, as both inorganic and organic mercurials have been reported to accumulate in the organelle. Cytochrome c was shown to leak from the mitochondria, followed by caspase 9 cleavage within 8 h of treatment. In addition, poly(ADP-ribose) polymerase (PARP) was cleaved to form a 85 kDa fragment following maximal caspase 3 activation at 24 h. Taken together these findings suggest deleterious effects on the cytoarchitecture by thimerosal and initiation of mitochondrial-mediated apoptosis.

Keywords: Mercury; Thimerosal; Mitochondria; Neurotoxicity

INTRODUCTION

Thimerosal (thiomersal, merthiolate, sodium ethylmercury thiosalicylate), an ethyl mercury-containing antibacterial and antifungal agent, has been used as an antiseptic and preservative in various formulations including paints, topical medications, eye lens cleaners and cosmetics since the 1930s. Concern has been raised by the use of thimerosal in more than 30 vaccines licensed in the United States (Elferink, 1999). With the addition of several important vaccines over the last decade and the use of multi-dose vials, exposure to

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Neuroligin-deficient mutants of *C. elegans* have sensory processing deficits and are hypersensitive to oxidative stress and mercury toxicity

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SUMMARY

Neuroligins are postsynaptic cell adhesion proteins that bind specifically to presynaptic membrane proteins called neurexins. Mutations in human neuroligin genes are associated with autism spectrum disorders in some families. The nematode *Caenorhabditis elegans* has a single neuroligin gene (*nlg-1*), and approximately a sixth of *C. elegans* neurons, including some sensory neurons, interneurons and a subset of cholinergic motor neurons, express a neuroligin transcriptional reporter. Neuroligin-deficient mutants of *C. elegans* are viable, and they do not appear deficient in any major motor functions. However, neuroligin mutants are defective in a subset of sensory behaviors and sensory processing, and are hypersensitive to oxidative stress and mercury compounds; the behavioral deficits are strikingly similar to traits frequently associated with autism spectrum disorders. Our results suggest a possible link between genetic defects in synapse formation or function, and sensitivity to environmental factors in the development of autism spectrum disorders.

INTRODUCTION

Neuroligins are a family of postsynaptic cell adhesion proteins that were originally isolated on the basis of their binding to presynaptic proteins called neurexins (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Boucard et al., 2005; Chih et al., 2006). Although early studies demonstrated that, under certain conditions, the interaction between neuroligin and neurexin was capable of inducing synaptogenesis (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004), recent studies suggest that neuroligins function primarily in the maturation, stability and/or maintenance of synapses, rather than synaptogenesis per se (Varoqueaux et al., 2006; Südhof, 2008).

There are four neuroligin genes in mammals, and several important studies have shown that mutations in

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Plenary article

Embryonic exposure to thimerosal, an organomercury compound, causes abnormal early development of serotonergic neurons

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ABSTRACT

Even though neuronal toxicity due to organomercury compounds is well known, thimerosal, an organomercury compound, is widely used in pediatric vaccine preservation. In the present study, we examined whether embryonic exposure to thimerosal affects early development of serotonergic neurons. Thimerosal (1 mg Hg/kg) was intramuscularly administered to pregnant rats on gestational day 9 (susceptible time window for development of fetal serotonergic system), and fetal serotonergic neurons were assessed at embryonic day 15 using anti-serotonin antibodies. A dramatic increase in the number of serotonergic neurons localized to the lateral portion of the caudal raphe was observed in thimerosal group (1.9-fold increase, $p < 0.01$ compared to control). These results indicate that embryonic exposure to thimerosal affects early development of serotonergic neurons.

Thimerosal, an organomercury compound, is known for its preservative effects on pediatric vaccines [2,12]. Thimerosal biotransforms *in vivo* to ethylmercury and subsequently into inorganic forms of mercury [19,20], which are toxic to animals [4,7]. Therefore, accumulation of mercury through frequent vaccine administration is a concern [2,26].

The adverse effects of thimerosal have been studied extensively; neonatal administration of thimerosal induces impairment of sensitivity to pain [16] and neurodegeneration of hippocampus [17]. Although fetal organomercury poisoning (fetal Minamata disease) is known to exhibit systemic effects on fetus [5,6], little is known regarding the mechanism of action of thimerosal during the embryonic period.

Serotonergic neurons are one of the earliest neurotransmitter phenotypes to appear during the development of the nervous system [1,8,10]. In the fetal rat, serotonergic neurons were identified at around embryonic day (E) 13 (day of insemination = E1) [1,18]. However, precursor cells that were fated to serotonergic neurons are known to appear, at the latest, by E9 [25]. We previously reported that E9 is the most critical time window for early development of serotonergic neurons [15], because exposure of pregnant rats to thalidomide resulted in caudal shift of serotonergic neurons in the dorsal raphe, suggestive of perturbed neuronal migration [13]. The effect of thalidomide was specific for the day of thalidomide administration, demonstrating that embryonic exposure at E9 is specifically crucial in the normal development of serotonergic neurons.

Since the early development of serotonergic neurons is time specific and three-dimensional [1,8,10], precise evaluation of serotonergic neuronal development by conventional immunohistochemical methods is difficult. In the present study, we utilized whole-mount preparation method for embryonic brain [1,9], which facilitates assessment of spatiotemporal data on the development of neurotransmitter system. Using this technique, we investigated whether exposure to thimerosal at E9 affects early development of serotonergic neurons.

Thimerosal administration: Pregnant Wistar rats were purchased by CLEA Japan, Inc. (Tokyo, Japan). Thimerosal (Sigma–Aldrich, St. Louis, MO) dissolved in saline (1 mg Hg/kg) was administrated into pregnant rats on gestational day 9 in volume of 50 μl, by intramuscular injection into *glutei maximi*. For control group, saline was administrated in the same way. Three dams for each group (thimerosal vs control) were examined. All animal experiments were authorized by the Animal Research Committee in Mie University.

Flat whole-mount preparation of rat brain: The procedure for preparing flat whole-mount hindbrain has been described previously [1,9,21,22]. In brief, E15 fetuses were dissected out and the

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Original article

Prenatal exposure to organomercury, thimerosal, persistently impairs the serotonergic and dopaminergic systems in the rat brain: Implications for association with developmental disorders

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Abstract

Thimerosal, an organomercury compound, has been widely used as a preservative. Therefore, concerns have been raised about its neurotoxicity. We recently demonstrated perturbation of early serotonergic development by prenatal exposure to thimerosal (Ida-Eto et al. (2011) [11]). Here, we investigated whether prenatal thimerosal exposure causes persistent impairment after birth. Analysis on postnatal day 50 showed significant increase in hippocampal serotonin following thimerosal administration on embryonic day 9. Furthermore, not only serotonin, striatal dopamine was significantly increased. These results indicate that embryonic exposure to thimerosal produces lasting impairment of brain monoaminergic system, and thus every effort should be made to avoid the use of thimerosal.

Keywords: Thimerosal; Serotonin; Dopamine; Embryonic exposure; Developmental disorders; Rat

1. Introduction

Thimerosal, an organomercury compound, has been widely used as a preservative [1]. Thimerosal is metabolized first to ethylmercury and further to inorganic mercury, both of which accumulate in the brain and other organs and have neurotoxic activity [2,3]. Accordingly, use of thimerosal such as vaccines is of great concern, particularly on infants and fetuses [4,5], and therefore, efforts have been made to reduce thimerosal from vaccines [6].

The adverse effects of thimerosal after neonatal administration include impaired pain sensitivity [7], hippocampal neurodegeneration [8], and changes in the dopamine system with subsequent behavioral disorders [9]. In addition, thimerosal was shown to affect neurite extension of neuroblastoma cells *in vitro*, therefore, it is evident that thimerosal leads to neurological abnormalities [10]. However, little is known regarding the prenatal effects of thimerosal. We recently reported that exposure of pregnant rats at gestational day 9 (E9) to thimerosal increased the number of serotonergic neurons in the lateral portion of the caudal raphe in E15 rat hindbrain and thus prenatal thimerosal exposure impaired early serotonergic development [11]. We have also demonstrated that prenatal exposure at E9 to thalidomide or valproic acid (VPA) specifically caused long-term effects on the normal development of serotonergic neuronal systems [12,13], accompanied with behavioral abnormalities that mimicked human

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Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated K^+ channel in the pancreatic β-cell

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Effects of sulfhydryl modification on the ATP regulated K^+ channel (K_{ATP} channel) in the pancreatic β-cell were studied, using the patch clamp technique. Application of the sulfhydryl oxidizing agents thimerosal and 2,2′-dithio-bis(5-nitropyridine) (DTBNP), in micromolar concentrations, caused complete inhibition of the K_{ATP} channel, in inside-out patches. The inhibition was rapid and was reversed by the disulfide reducing agents dithiothreitol and cysteine. Thimerosal, which is poorly membrane permeable, inhibited channel activity, only when applied to the intracellular face of the plasma membrane. In contrast, DTBNP, which is highly lipophilic, caused closure of the K_{ATP} channel and consequent depolarization of the membrane potential, also when applied extracellularly. Our results indicate the presence of accessible free SH groups on the cytoplasmic side of the K_{ATP} channel in the pancreatic β-cell. These SH groups are essential for channel function and it is possible that thiol-dependent redox mechanisms can modulate K_{ATP} channel activity.

ATP-regulated K^+ channel; Sulfhydryl reagent; Thimerosal; Pancreatic β-cell

1. INTRODUCTION

K^+ channels characterized by their sensitivity to intracellular ATP (K_{ATP}), play an important role in the regulation of insulin secretion from the pancreatic β-cell [1–3]. Under resting conditions, at glucose concentrations less than 5 mM, the K_{ATP} conductance dominates and therefore determines the membrane potential of the β-cell [4]. A key event in the glucose stimulation of insulin secretion is the closure of this channel. Closure of the K_{ATP} channel results in depolarization of the cell, Ca^{2+}-influx through the voltage-gated Ca^{2+} channel, increase in the cytoplasmic free Ca^{2+} concentration and insulin secretion [3,5]. The K_{ATP} channel is also the target for sulfonylureas, a class of drugs which inhibits channel activity, and are used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) [6]. The precise signals that generate from glucose metabolism and control the activity of the K_{ATP} channel are still unknown. Currently, a change in the intracellular concentration of ATP or ATP/ADP ratio is believed to be the most important link between fuel metabolism and depolarization of the cell [2,7]. However the regulation of the channel appears to be more complex than that and may involve modulation by protein kinase C, G proteins and changes in the redox potential of the cell. [8–13]. At present little is known about the structure of the K_{ATP} channel protein, as well as about the molecular basis of its regulation.

Many biologically active proteins contain critical cysteine residues. The function of these proteins often depends on the oxidation state of sulfhydryl (thiol) groups (SH groups) [14]. Some proteins are active only when their specific SH groups remain in the reduced form, whereas for the activity of others the disulfide redox state is essential [15,16]. Selective modification of SH groups, has been extensively used to ascertain the relationship between structure and function of many biomolecules. Different types of ion channel proteins also contain SH groups, modification of which may affect channel activity [17,18]. The sulfhydryl reagent thimerosal and some 'reactive disulfides' open intracellular Ca^{2+} channels by oxidizing critical SH groups [18,19]. There is evidence to suggest, that the K_{ATP} channel of mouse skeletal muscle contains functionally important SH groups [20]. The role of SH groups in regulating the activity of the K_{ATP} channel in the pancreatic β-cell is unknown, although it is known since long that many sulfhydryl reagents stimulate insulin secretion [21–24]. In the present study we demonstrate that the sulfhydryl oxidizing agents, thimerosal and 2,2′-dithio-bis(5-nitropyridine) (DTBNP) induce rapid and reversible closure of the K_{ATP} channel in the pancreatic β-cell, indicating that this channel contains SH groups essential for the channel activity.

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

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Abstract

Thimerosol 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 thimerosol 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 μM 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 μM 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 or NAC against mercury toxicity warrants further research as possible adjunct therapy to individuals still receiving Thimerosal-containing vaccinations.

Keywords: Thimerosal; Neurotoxicity; Glutathione; *N*-acetylcysteine

INTRODUCTION

Thimerosal (sodium ethylmercurithiosalicylate) was developed by Eli Lilly in the 1930s as a 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 (μg/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 μg/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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Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism

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ABSTRACT **Research into the metabolic phenotype of autism has been relatively unexplored despite the fact that metabolic abnormalities have been implicated in the pathophysiology of several other neurobehavioral disorders. Plasma biomarkers of oxidative stress have been reported in autistic children; however, intracellular redox status has not yet been evaluated. Lymphoblastoid cells (LCLs) derived from autistic children and unaffected controls were used to assess relative concentrations of reduced glutathione (GSH) and oxidized disulfide glutathione (GSSG) in cell extracts and isolated mitochondria as a measure of intracellular redox capacity. The results indicated that the GSH/GSSG redox ratio was decreased and percentage oxidized glutathione increased in both cytosol and mitochondria in the autism LCLs. Exposure to oxidative stress *via* the sulfhydryl reagent thimerosal resulted in a greater decrease in the GSH/GSSG ratio and increase in free radical generation in autism compared to control cells. Acute exposure to physiological levels of nitric oxide decreased mitochondrial membrane potential to a greater extent in the autism LCLs, although GSH/GSSG and ATP concentrations were similarly decreased in both cell lines. These results suggest that the autism LCLs exhibit a reduced glutathione reserve capacity in both cytosol and mitochondria that may compromise antioxidant defense and detoxification capacity under prooxidant conditions.—James, S. J., Rose, S., Melnyk, S., Jernigan, S., Blossom, S., Pavliv, O., Gaylor, D. W. Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. *FASEB J.* 23, 2374–2383 (2009)**

Key Words: autistic disorder · oxidative stress · nitric oxide

Autism is a behaviorally defined neurodevelopmental disorder characterized by impairments in social interaction and communication skills and by hyperfocused interests and compulsive behaviors. Autism is usually diagnosed before 4 yr of age and is estimated to affect 1 in 150 children in the United States, with a 4:1 male to female gender bias (1). Although multiple interacting genetic and environmental factors are thought to influence individual vulnerability to autism, none have been reproducibly identified in more than a fraction of cases. In addition to complex gene-environment interactions, the heterogeneous presentation of behavioral symptoms within the spectrum of autistic disorders suggests a variable and multifactorial pathogenesis.

Several lines of evidence suggest that underlying oxidative stress and glutathione depletion contribute to pathophysiology of several neurobehavioral disorders, including schizophrenia (2, 3), bipolar disorder (4, 5), Parkinson's disease (6, 7), Alzheimer's disease (8, 9), and autism. Children with autism have been shown to exhibit evidence of lipid peroxidation (10, 11), reduced antioxidant activity (10, 12, 13), elevated nitric oxide levels (14, 15), and accumulation of advanced glycation end products (AGEs) and the proinflammatory AGE receptor ligand S100A9 (16). The presence of redox imbalance and chronic oxidative stress in autism is further supported by evidence of microglial inflammation (17) and decreased glutathione-mediated redox status (18, 19). Although provocative, it is not clear whether these measures of oxidative stress are present during early development and contribute to pathogenesis of autism, or whether they are a secondary manifestation of the disorder.

Oxidative stress is traditionally defined as an imbalance between oxidant generation and antioxidant defense mechanisms that leads to macromolecular damage and dysfunction. More recently, the definition has expanded to include more subtle perturbations in redox signaling mechanisms that control and regulate a wide variety of cellular functions, including enzyme activation/inhibition (20, 21), membrane signal transduction (22, 23), transcription factor binding/gene expression (24, 25), proliferation/apoptosis (26–28), and precursor cell ontogeny (29, 30). The ratio of reduced glutathione (GSH) to the oxidized disulfide form of glutathione (GSSG) is considered a reproduc-

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Thimerosal decreases TRPV1 activity by oxidation of extracellular sulfhydryl residues

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Abstract

TRPV1, a receptor for capsaicin, plays a key role in mediating thermal and inflammatory pain. Because the modulation of ion channels by the cellular redox state is a significant determinant of channel function, we investigated the effects of sulfhydryl modification on the activity of TRPV1. Thimerosal, which oxidizes sulfhydryls, blocked the capsaicin-activated inward current (I_{cap}) in cultured sensory neurons, in a reversible and dose-dependent manner, which was prevented by the co-application of the reducing agent, dithiothreitol. Among the three cysteine residues of TRPV1 that are exposed to the extracellular space, the oxidation-induced effect of thimerosal on I_{cap} was blocked only by a point mutation at Cys621. These results suggest that the modification of an extracellular thiol group can alter the activity of TRPV1. Consequently, we propose that such a modulation of the redox state might regulate the physiological activity of TRPV1.

Keywords: Thimerosal; Sulfhydryl oxidation; Capsaicin; TRPV1; Dorsal root ganglion

Capsaicin, the main pungent ingredient of hot peppers, produces burning pain and neurogenic inflammation through the excitation of small sensory neurons [3,25]. In cultured dorsal root ganglion (DRG) neurons, capsaicin activates a ligand-gated, non-selective cation channel [19]. A cDNA that encodes a channel that is activated by capsaicin was cloned recently, and was classified as TRPV1 [4,18]. The TRPV1 channel has properties that resemble those of the capsaicin-activated channel that is present in sensory neurons. In addition, TRPV1 is activated by heat and extracellular acid [4,27], and the lipid metabolic products of lipoxygenases and anandamide activate TRPV1 [13,23,31]. TRPV1-deficient mice exhibit reduced inflammatory thermal hyperalgesia, so TRPV1 appears to be essential for mediating thermal hyperalgesia that is induced by inflammation [5,6].

During inflammation or reperfusion injury, reactive oxygen species are generated, which affects the redox state of tissues [11]. Among the many amino acids that are present within biologically active proteins, cysteine residues are reactive to the cellular redox state. Thus, redox modification of cysteinyl sulfhydryl (SH) groups due to a change in the redox state constitute an important mechanism for regulating cellular function. Oxidation of cysteine residues modulates the activity of the channels in *N*-methyl-D-aspartate receptors [2], $GABA_A$ receptors [1], ATP-sensitive K^+ channels [20], large conductance Ca^{2+}-activated K^+ channels [7], and voltage-dependent Ca^{2+} [17,26] and Na^+ channels [24].

Recently, intradermal injection of a reducing agent, dithiothreitol (DTT), was reported to induce thermal hyperalgesia, an effect that could be blocked by co-injection of an oxidizing agent, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) [26]. These results suggested that changes in the redox state in peripheral tissues may influence the activity of ion channels that are expressed in sensory neurons. Because TRPV1 has been implicated in mediating inflammatory pain [5,6], it is possible that changes in the redox state in peripheral tissues modulate the activity of TRPV1. In the present study, we examined the effects of a sulfhydryl-oxidizing agent, thimerosal, on TRPV1-dependent currents in cultured DRG neurons. In ad-

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Purified Reconstituted Inositol 1,4,5-Trisphosphate Receptors

THIOL REAGENTS ACT DIRECTLY ON RECEPTOR PROTEIN*

(Received for publication, March 18, 1994, and in revised form, August 15, 1994)

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Thimerosal, a sulfhydryl oxidizing reagent, has been shown to induce Ca^{2+} mobilization in several cell types and to increase the sensitivity of intracellular Ca^{2+} stores to inositol 1,4,5-trisphosphate (IP_3). Using purified IP_3 receptor (IP_3R) protein reconstituted in vesicles, we demonstrate pronounced stimulation by thimerosal of its Ca^{2+} channel activity. Effects of thimerosal are dependent on the redox state of the receptor, implying an action of thimerosal on a critical sulfhydryl group(s) of IP_3R. Thimerosal enhances the affinity of IP_3R for IP_3 binding. The manner in which thimerosal modulates IP_3R responsiveness to IP_3 provides evidence for receptor heterogeneity with implications for mechanisms of quantal Ca^{2+} release. These results clarify regulation of IP_3R activity by redox modulation.

The dynamics of intracellular Ca^{2+} provide crucial signaling information in many aspects of cellular regulation. Intracellular Ca^{2+} flux is regulated by numerous processes, especially the release of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate (IP_3)[1] (1,2) and a calcium-induced calcium release system involving channels labeled by the alkaloid ryanodine (3, 4). Physiologic and pathologic changes in the oxidative state of cells influence Ca^{2+} disposition. Low levels of oxidants can stimulate cell proliferation and differentiation (5, 6), while increased oxidative stress may exert cytotoxic effects, including death by apoptosis (7). Perturbations in the oxidative state of sulfhydryl groups can influence Ca^{2+} flux (7). Thimerosal (TMS), a sulfhydryl oxidizing agent, has been reported to stimulate (8–12) or inhibit (11, 12) intracellular Ca^{2+} flux. TMS increases the potency of IP_3 in releasing Ca^{2+} (9, 11, 13–16). In some studies, TMS increased the affinity of IP_3 for receptor binding sites (13, 16), while other studies showed no effect (11, 12). These findings suggest that IP_3-induced Ca^{2+} flux is influenced by the oxidative state of sulfhydryl groups, perhaps those of the IP_3 receptor (IP_3R) itself. However, TMS can influence related Ca^{2+} regulating systems, such as the endoplasmic reticular Ca^{2+} pump (11, 13), indirectly affecting IP_3 induced Ca^{2+} release.

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[1] The abbreviations used are: IP_3, inositol 1,4,5-trisphosphate; TMS, thimerosal; IP_3R, IP_3 receptor; IP_3RV, IP_3 reconstitutes vesicles; BME, B-mercaptoethanol; DTT, dithiothreitol; IAA, iodoacetamide; NEM, *N*-ethylmaleimide.

We successfully reconstituted IP_3 induced Ca^{2+} flux in vesicles containing only purified IP_3R protein (17). IP_3-mediated Ca^{2+} flux in the reconstituted vesicles is regulated by phosphorylation (18, 19) and adenine nucleotides (20). Using these reconstituted vesicles, we have characterized effects of thiol reagents on Ca^{2+} flux at the level of the IP_3R protein.

Intracellular release of Ca^{2+} is a discontinuous, quantal process in which successive increments of IP_3 transiently release precise amounts of Ca^{2+} (21, 22). Utilizing IP_3R reconstituted into proteolipid vesicles (IP_3RV) we previously showed that quantal flux of Ca^{2+} elicited by IP_3 is a fundamental property of the IP_3R, suggesting that the receptors purified from rat cerebellum constitute a heterogeneous population with varying sensitivity to IP_3 (23). The effects of thiol reagents on IP_3-mediated flux in IP_3RV observed here provide additional evidence for functional receptor heterogeneity, which may help account for quantal Ca^{2+} release.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]IP_3, $^{45}Ca^{2+}$, and formula 963 scintillation mixture were obtained from DuPont NEN. D-*myo*-Ins(1,4,5)P_3, hexapotassium salt was obtained from LC Laboratories (Woburn, MA). Concanavalin A-Sepharose and G-25, superfine, were obtained from Pharmacia LKB Biotechnology Inc. Phospholipids for reconstitution were obtained from Avanti Polar Lipids (Birmingham, AL). All other reagents were from Sigma.

Purification and Reconstitution of IP_3R—IP_3R was purified from adult male Sprague-Dawley rat cerebellum and reconstituted into lipid vesicles as described (17). Briefly, IP_3R was purified using a two-step affinity chromatography procedure employing sequential heparin-agarose and concanavalin A-Sepharose columns. Following purification to apparent homogeneity, detergent-solubilized receptor protein was mixed with sonicated lipids and the mixture was dialyzed at 4 °C against buffer A (50 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, pH 7.4), supplemented with 2.5 mM B-mercaptoethanol (BME) and 2 mM EDTA, to effect detergent removal and vesicle formation. The buffer was changed every 8 h for 48 h, and EDTA was omitted from the final buffer change. For experiments performed in the absence of reducing agent, 1 ml of IP_3RV was passed over a 5-ml G-25 desalting column equilibrated with buffer A to remove BME.

$^{45}Ca^{2+}$ Flux—Reconstituted proteoliposomes were assayed for IP_3-stimulated $^{45}Ca^{2+}$ flux as described (17). Following preincubation under various conditions vesicles were incubated (for either 10 or 15 s) in the presence of 2 μCi of $^{45}Ca^{2+}$ with or without IP_3. Under these conditions, tracer $^{45}Ca^{2+}$ gained access to the lumen of vesicles when the IP_3R channels were opened by IP_3. The flux reaction was stopped by the addition of excess buffer containing unlabeled divalent cations and heparin (200 μg/ml). Intravesicular $^{45}Ca^{2+}$ content was isolated by immediately passing the vesicle/buffer mixture over a cation-exchange column (Dowex 50W, Sigma). The vesicles were collected and their intravesicular $^{45}Ca^{2+}$ content was measured by scintillation spectrometry.

[^{3}H]IP_3 Binding—Ligand binding was assayed by precipitation of IP_3RV with polyethylene glycol using γ-globulin as carrier protein, as described (20).

RESULTS

Several workers have shown TMS stimulation of Ca^{2+} flux in intact cells and platelets (14, 15, 24–29) and enhancement of

Case Report

Apparent vaccine-thimerosal induced hypersensitivity, myelodysplastic syndrome and pancytopenia

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Abstract
A case of hypersensitivity reaction, myelodysplastic syndrome and pancytopenia, which developed after an administration of thimerosal-containing tetanus vaccine, is presented and discussed.
Key Words: Thimerosal, hypersensitivity reaction, myelodysplastic syndrome, pancytopenia.

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Introduction

Thimerosal is used as a preservative for many vaccines, and so is one of the most important organic mercury compounds to which human populations are exposed. Despite evidence of toxicity, the World Health Organization (WHO) continues to recommend the use of thimerosal-containing vaccines, especially those available in multidose vials, in countries that do not have resources for alternative vaccine preparations because the benefit-cost ratio remains very high [1]. The following report presents an account of probable thimerosal toxicity induced by vaccination in an adult woman.

Case

A forty-three-year-old woman was admitted to our department on May 1, 2006, with a history of tetanus vaccination one month previously. Immediately following vaccination she experienced difficulty in breathing, chest tightness, headache and facial flushing. Swelling started in the periorbital area, and over the next 3 days spread to her shoulders and arms, eventually affecting the whole body. She reported general fatigue and malaise and noted a jaundiced appearance. These symptoms decreased within one month but did not completely disappear. When she was referred to our department one month later, she had still facial swelling and flushing, together with jaundice and profound weakness and fatigue.

Physical examination revealed moderate general status, ill appearance, with facial swelling and flushing, especially in the periorbital region. Her blood pressure was 110/80 mmHg, pulse rate 86/minute, and respiration rate 20/minute; other systems were normal. Urinalysis was normal. Haematological findings were WBC 2600 /mm³, hemoglobin 4.2, g/dl, hematocrit 16%, MCV 60, platelets 53.000 /mm³, PT 13 sec, and PTT 34 sec, fibrinogen 259 mg/dl, ferritin <1.5 ng/ml, serum iron 14.4 μg/dl, serum iron binding capacity 287 μg/dl, vitamin B12 379 pg/ml, folate 5 ng/ml. Blood biochemistry showed ALT 12 U/L, AST 14 U/L and AFP 0.8 ng/ml. Faecal examination showed no occult blood or parasites. Serological examination for a wide variety of bacterial and viral infections showed only a positive HAV IgG, and malignancy and autoimmune markers were negative. Abdominal ultrasonography showed normal liver size, but the liver parenchyma had a heterogeneous appearance with a granular pattern and liver biopsy showed non-specific hepatitis. Changes consistent with myelodysplastic syndrome were observed in a bone marrow biopsy.

The vaccine given was produced at the Serum Institute of India Ltd and contained the standard 0.01% thimerosal as a preservative against

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RESEARCH Open Access

Mercury induces inflammatory mediator release from human mast cells

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Abstract

Background: Mercury is known to be neurotoxic, but its effects on the immune system are less well known. Mast cells are involved in allergic reactions, but also in innate and acquired immunity, as well as in inflammation. Many patients with Autism Spectrum Disorders (ASD) have "allergic" symptoms; moreover, the prevalence of ASD in patients with mastocytosis, characterized by numerous hyperactive mast cells in most tissues, is 10-fold higher than the general population suggesting mast cell involvement. We, therefore, investigated the effect of mercuric chloride ($HgCl_2$) on human mast cell activation.

Methods: Human leukemic cultured LAD2 mast cells and normal human umbilical cord blood-derived cultured mast cells (hCBMCs) were stimulated by HgCl2 (0.1-10 μM) for either 10 min for beta-hexosaminidase release or 24 hr for measuring vascular endothelial growth factor (VEGF) and IL-6 release by ELISA.

Results: $HgCl_2$ induced a 2-fold increase in β-hexosaminidase release, and also significant VEGF release at 0.1 and 1 μM (311 ± 32 pg/10^6 cells and 443 ± 143 pg/10^6 cells, respectively) from LAD2 mast cells compared to control cells (227 ± 17 pg/10^6 cells, n = 5, $p < 0.05$). Addition of $HgCl_2$ (0.1 μM) to the proinflammatory neuropeptide substance P (SP, 0.1 μM) had synergestic action in inducing VEGF from LAD2 mast cells. $HgCl_2$ also stimulated significant VEGF release (360 ± 100 pg/10^6 cells at 1 μM, n = 5, $p < 0.05$) from hCBMCs compared to control cells (182 ± 57 pg/10^6 cells), and IL-6 release (466 ± 57 pg/10^6 cells at 0.1 μM) compared to untreated cells (13 ± 25 pg/10^6 cells, n = 5, $p < 0.05$). Addition of $HgCl_2$ (0.1 μM) to SP (5 μM) further increased IL-6 release.

Conclusions: $HgCl_2$ stimulates VEGF and IL-6 release from human mast cells. This phenomenon could disrupt the blood-brain-barrier and permit brain inflammation. As a result, the findings of the present study provide a biological mechanism for how low levels of mercury may contribute to ASD pathogenesis.

Background

Heavy metals such as mercury result in neurological injury that may lead to developmental defects, peripheral neuropathies, and enhanced neurodegenerative changes [1]. Mercurials may be found in various drugs, in bleaching creams, antiseptics, disinfectants, as preservatives in cosmetics, tooth pastes, lens solutions, vaccines, contraceptives and immunotherapy solutions, fungicides, herbicides and in dental fillings, as well as in fish such as tuna due to water pollution [2]. Mercury can cause immune, sensory, neurological, motor, and behavioral dysfunction similar to those associated with Autism Spectrum Disorders (ASD) [2]. The possible role of mercury used as preservative in vaccines [2] has been debated extensively, but most epidemiological studies do not support a causal association between vaccines and autism [3-7]. However, 87% of children included in the US Vaccine Adverse Event Reporting System (VAERS) had ASD [8]. Moreover, a paper based on computerized medical records in the Vaccine Safety Data-link concluded there was "significantly increased rate ratios for ASD with mercury exposure from thiomerosal-containing vaccines" [9]. Mercury has been shown to induce proliferation and cytokine production from T lymphocytes [10]. Mercuric chloride ($HgCl_2$) in nontoxic doses induces the release of histamine and cytokines, such as IL-4 and tumor necrosis factor-alpha (TNF-α), from a

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A biomarker of mercury body-burden correlated with diagnostic domain specific clinical symptoms of autism spectrum disorder

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Abstract The study purpose was to compare the quantitative results from tests for urinary porphyrins, where some of these porphyrins are known biomarkers of heavy metal toxicity, to the independent assessments from a recognized quantitative measurement, the Autism Treatment Evaluation Checklist (ATEC), of specific domains of autistic disorders symptoms (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) in a group of children having a clinical diagnosis of autism spectrum disorder (ASD). After a Childhood Autism Rating Scale (CARS) evaluation to assess the development of each child in this study and aid in confirming their classification, and an ATEC was completed by a parent, a urinary porphyrin profile sample was collected and sent out for blinded analysis. Urinary porphyrins from twenty-four children, 2–13 years of age, diagnosed with autism or PDD-NOS were compared to their ATEC scores as well as their scores in the specific domains (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) assessed by ATEC. Their urinary porphyrin samples were evaluated at Laboratoire Philippe Auguste (which is an ISO-approved clinical laboratory). The results of the study indicated that the participants' overall ATEC scores and their scores on each of the ATEC subscales (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) were linearly related to urinary porphyrins associated with mercury toxicity. The results show an association between the apparent level of mercury toxicity as measured by recognized urinary porphyrin biomarkers of mercury toxicity and the magnitude of the specific hallmark features of autism as assessed by ATEC.

Keywords Toxicity · Mercury · CARS · ATEC · ASDs · Asperger's · Autism · PDD-NOS · Porphyrins · Biomarkers · Susceptibility

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Toxicity biomarkers among US children compared to a similar cohort in France: a blinded study measuring urinary porphyrins

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The purpose of this blinded study was to evaluate potential environmental toxicity in a cohort of neurotypical children ($n = 28$) living in a suburban area of north-central Texas in the United States (US) with a comparable age- and gender-matched cohort of neurotypical children ($n = 28$) living in a suburban area of southeastern France using urinary porphyrin testing: uroporphyrin (uP), heptacarboxyporphyrin (7cxP), hexacarboxyporphyrin (6cxP), pentacarboxyporphyrin (5cxP), precoproporphyrin (prcP), and coproporphyrin (cP). Results showed significantly elevated 6cxP, prcP (an atypical, mercury-specific porphyrin), and cP levels, and increasing trends in 5cxP levels, among neurotypical children in the USA compared to children in France. Data suggest that in US neurotypical children, there is a significantly increased body-burden of mercury (Hg) compared to the body-burden of Hg in the matched neurotypical children in France. The presence of lead contributing to the higher levels of cP also needs to be considered. Further, other factors including genetics can not be completely ruled out.

Keywords: mercury; heavy metal; porphyrins; biomarkers; xenobiotic; lead; toxicity

Introduction

For many years, measuring heavy metal toxicity in children involved a direct measure of the metals in the blood, urine, hair, or fecal matter. A more recent approach is to use urinary porphyrins as a measure of toxic metal body-burden. Previous studies showed that urinary porphyrins (heme precursors formed in the heme synthesis pathway) afford a measure of xenobiotic exposure, particularly mercury (Hg) (Woods 1996; Pingree et al. 2001a; Pingree, Simmonds, and Woods 2001b). Specific patterns of porphyrins suggest the presence of Hg exposure. Mercury toxicity was demonstrated to be associated with

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Original Article

Toxicity biomarkers in autism spectrum disorder: A blinded study of urinary porphyrins

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Abstract ***Background***: Recent studies suggest that children diagnosed with an autism spectrum disorder (ASD) have significantly increased levels of urinary porphyrins associated with mercury (Hg) toxicity, including pentacarboxyporphyrin (5cxP), precoproporphyrin (prcP), and coproporphyrin (cP), compared to typically developing controls. However, these initial studies were criticized because the controls were not age- and gender-matched to the children diagnosed with an ASD.
Methods: Urinary porphyrin biomarkers in a group of children (2–13 years of age) diagnosed with an ASD ($n = 20$) were compared to matched (age, gender, race, location, and year tested) group of typically developing controls ($n = 20$).
Results: Participants diagnosed with an ASD had significantly increased levels of 5cxP, prcP, and cP in comparison to controls. No significant differences were found in non-Hg associated urinary porphyrins (uroporphyrins, hexacarboxyporphyrin, and heptacarboxyporphyrin). There was a significantly increased odds ratio for an ASD diagnosis relative to controls among study participants with precoproporphyrin (odds ratio = 15.5, $P < 0.01$) and coproporphyrin (odds ratio = 15.5, $P < 0.01$) levels in the second through fourth quartiles in comparison to the first quartile.
Conclusion: These results suggest that the levels of Hg-toxicity-associated porphyrins are higher in children with an ASD diagnosis than controls. Although the pattern seen (increased 5cxP, prcP, and cP) is characteristic of Hg toxicity, the influence of other factors, such as genetics and other metals cannot be completely ruled out.

Key words autism, autism spectrum disorder, heavy metal, mercury, porphyrins, toxicity.

Introduction

An autism spectrum disorder (ASD) is a neurological disorder that limits a person's ability to function normally. Behavioral abnormalities, social limitations, sensory processing abnormalities, and impaired ability to communicate are the main issues in these multifaceted disorders, which range in clinical symptoms, from severe to mild among individuals diagnosed with autistic disorder (autism), pervasive developmental delay not otherwise defined (PDD-NOS), to Asperger's disorder.[1,2]

Although the role of mercury (Hg) in the pathology of autism is still being debated, many studies suggest that Hg levels are higher in children with autism that in typically developing children (controls), e.g. studies that examine Hg levels in hair, blood, urine, and teeth.[3] A more recent approach is to use urinary porphyrins as measure of Hg body-burden. Previous studies have shown that urinary porphyrins (heme precursors formed in the heme synthesis pathway, Fig. 1) can afford a measure of xenobiotic exposure, particularly Hg.[5–7] Specific patterns of urinary porphyrins suggest the presence of Hg. Hg toxicity has been demonstrated to be associated with elevations in urinary coproporphyrin (cP), pentacarboxyporphyrin (5cxP), and by the expression of an atypical porphyrin–precoproporphyrin (prcP) (also known as keto-isocoproporphyrin) not found in the urine of unexposed controls. Woods[5] noted that these distinct changes in urinary porphyrin concentrations were observed as early as 1–2 weeks after initiation of Hg exposure, and that they increased in a dose- and time-related fashion with the concentration of Hg in the kidney, a principal target organ of Hg compounds. In addition, urinary porphyrin profiles were also shown to correlate significantly with Hg body-burden and with specific neurobehavioral deficits associated with low level Hg exposure. Woods[5] concluded that urinary porphyrin profiles are a useful biomarker for Hg exposure and its potential adverse health effects in human subjects.

Recent evidence suggests that the levels of Hg-associated porphyrins are different in children having a diagnosis of an ASD as compared to those levels in controls. Studies revealed that children with an ASD diagnosis had significantly increased levels

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Evidence of parallels between mercury intoxication and the brain pathology in autism

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The purpose of this review is to examine the parallels between the effects mercury intoxication on the brain and the brain pathology found in autism spectrum disorder (ASD). This review finds evidence of many parallels between the two, including: (1) microtubule degeneration, specifically large, long-range axon degeneration with subsequent abortive axonal sprouting (short, thin axons); (2) dentritic overgrowth; (3) neuroinflammation; (4) microglial/astrocytic activation; (5) brain immune response activation; (6) elevated glial fibrillary acidic protein; (7) oxidative stress and lipid peroxidation; (8) decreased reduced glutathione levels and elevated oxidized glutathione; (9) mitochondrial dysfunction; (10) disruption in calcium homeostasis and signaling; (11) inhibition of glutamic acid decarboxylase (GAD) activity; (12) disruption of GABAergic and glutamatergic homeostasis; (13) inhibition of IGF-1 and methionine synthase activity; (14) impairment in methylation; (15) vascular endothelial cell dysfunction and pathological changes of the blood vessels; (16) decreased cerebral/cerebellar blood flow; (17) increased amyloid precursor protein; (18) loss of granule and Purkinje neurons in the cerebellum; (19) increased pro-inflammatory cytokine levels in the brain (TNF-α, IFN-γ, IL-1β, IL-8); and (20) aberrant nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This review also discusses the ability of mercury to potentiate and work synergistically with other toxins and pathogens in a way that may contribute to the brain pathology in ASD. The evidence suggests that mercury may be either causal or contributory in the brain pathology in ASD, possibly working synergistically with other toxic compounds or pathogens to produce the brain pathology observed in those diagnosed with an ASD.

Key words: autism, autism spectrum disorder (ASD), mercury (Hg), toxicity, brain pathology

INTRODUCTION

Evidence suggests that children with autism spectrum disorder (ASD) have a greater susceptibility to heavy-metal intoxication than typically developing children (Holmes et al. 2003, Kern and Jones 2006, Rose et al. 2008, Nataf et al. 2008, James et al. 2009, Geier et al. 2009a, Majewska et al. 2010, Youn et al. 2010, Kern et al. 2011a). For example, children with ASD have been found to have low plasma glutathione (GSH) and sulfate (SO_4) levels (Waring and Klovrza 2000, James et al. 2004, 2006, 2009, Geier and Geier 2006, Geier et al. 2009c, Pasca et al. 2009, Adams et al. 2011), both of which are critically important for detoxification (Gutman 2002, Kern et al. 2004). Expressions such as "poor detoxifiers" and "poor excretors" have been used in reference to those with ASD (Holmes et al. 2003). In a recent analysis, DeSoto and Hitlan (2010) found that there are 58 research articles which provide empirical evidence relevant to the question of a link between autism and one or more heavy metals. Of those 58 articles, 43 supported a statistically significant link between autism and exposure to toxic metals while 15 showed no statistically significant evidence of a link between metals and autism. Thus, 74% of the studies examined showed a significant relationship between ASD and toxic metals. Moreover, several recent studies have shown that the greater the toxic metal body burden in a child, the worse the autism symptoms that the child experiences

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REVIEW PAPER

Systematic Assessment of Research on Autism Spectrum Disorder and Mercury Reveals Conflicts of Interest and the Need for Transparency in Autism Research

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Abstract Historically, entities with a vested interest in a product that critics have suggested is harmful have consistently used research to back their claims that the product is safe. Prominent examples are: tobacco, lead, bisphenol A, and atrazine. Research literature indicates that about 80–90 % of studies *with* industry affiliation found no harm from the product, while only about 10–20 % of studies *without* industry affiliation found no harm. In parallel to other historical debates, recent studies examining a possible relationship between mercury (Hg) exposure and autism spectrum disorder (ASD) show a similar dichotomy. Studies sponsored and supported by industry or entities with an apparent conflict of interest have most often shown no evidence of harm or no "consistent" evidence of harm, while studies without such affiliations report positive evidence of a Hg/autism association. The potentially causal relationship between Hg exposure and ASD differs from other toxic products since there is a broad coalition of entities for whom a conflict of interest arises. These include influential governmental public health entities, the pharmaceutical industry, and even the coal burning industry. This review includes a systematic literature search of original studies on the potential relationship between Hg and ASD from 1999 to date, finding that of the studies with public health and/or industry affiliation, 86 % reported no relationship between Hg and ASD. However, among studies without public health and/or industry affiliation, only 19 % find no relationship between Hg and ASD. The discrepancy in these results suggests a bias indicative of a conflict of interest.

Keywords Research · Conflict of interest · Transparency · Autism · Mercury · Toxicants

Janet K. Kern, David A. Geier, Richard C. Deth, Lisa K. Sykes, Brian S. Hooker, James M. Love, Geir Bjørklund, Carmen G. Chaigneau, Boyd E. Haley, Mark R. Geier

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Review

The relationship between mercury and autism: A comprehensive review and discussion

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ABSTRACT

The brain pathology in autism spectrum disorders (ASD) indicates marked and ongoing inflammatory reactivity with concomitant neuronal damage. These findings are suggestive of neuronal insult as a result of external factors, rather than some type of developmental mishap. Various xenobiotics have been suggested as possible causes of this pathology. In a recent review, the top ten environmental compounds suspected of causing autism and learning disabilities were listed and they included: lead, methylmercury, polychorinated biphenyls, organophosphate pesticides, organochlorine pesticides, endocrine disruptors, automotive exhaust, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, and perfluorinated compounds. This current review, however, will focus specifically on mercury exposure and ASD by conducting a comprehensive literature search of original studies in humans that examine the potential relationship between mercury and ASD, categorizing, summarizing, and discussing the published research that addresses this topic. This review found 91 studies that examine the potential relationship between mercury and ASD from 1999 to February 2016. Of these studies, the vast majority (74%) suggest that mercury is a risk factor for ASD, revealing both direct and indirect effects. The preponderance of the evidence indicates that mercury exposure is causal and/or contributory in ASD.

Contents

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SEX-DEPENDENT CHANGES IN CEREBELLAR THYROID HORMONE-DEPENDENT GENE EXPRESSION FOLLOWING PERINATAL EXPOSURE TO THIMEROSAL IN RATS

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Mammalian brain development is regulated by the action of thyroid hormone (TH) on target genes. We have previously shown that the perinatal exposure to thimerosal (TM, metabolized to ethylmercury) exerts neurotoxic effects on the developing cerebellum and is associated with a decrease in cerebellar D2 activity, which could result in local brain T3 deficiency. We have also begun to examine TM effect on gene expression. The objective of this study was to expand on our initial observation of altered cerebellar gene expression following perinatal TM exposure and to examine additional genes that include both TH-dependent as well as other genes critical for cerebellar development in male and female neonates exposed perinatally (G10-G15 and P5 to P10) to TM. We report here for the first time that expression of suppressor-of-white-apricot-1 (SWAP-1), a gene negatively regulated by T3, was increased in TM-exposed males (61.1% increase), but not in females; ($p<0.05$). Positively regulated T3-target genes, Purkinje cell protein 2 (Pcp2; $p=0.07$) and Forkhead box protein P4 (FoxP4; $p=0.08$), showed a trend towards decreased expression in TM-exposed males. The expression of deiodinase 2 (DIO2) showed a trend towards an increase in TM-exposed females, while deiodinase 3 (DIO3), transthyretin (TTR), brain derived neurotrophic factor (BDNF) and reelin (RELN) was not significantly altered in either sex. Since regulation of gene splicing is vital to neuronal proliferation and differentiation, altered expression of SWAP-1 may exert wide ranging effects on multiple genes involved in the regulation of cerebellar development. We have previously identified activation of another TH-dependent gene, outer dense fiber of sperm tails 4, in the TM exposed male pups. Together, these results also show sex-dependent differences between the toxic impacts of TM in males and females. Interestingly, the genes that were activated by TM are negatively regulated by TH, supporting our hypothesis of local brain hypothyroidism being induced by TM and suggesting a novel mechanism of action TM in the developing brain.

Key words: *cerebellum, suppressor-of-white-apricot-1 (SWAP-1), thimerosal, thyroid hormone, brain derived neurotrophic factor, reelin*

INTRODUCTION

Thyroid hormone (TH) is critical for brain development; its deficiency during the perinatal period is associated with abnormalities in brain structure and function (1). Many factors, both genetic and environmental may contribute to TH deficiency. Of interest to this study is the contribution of TH-disrupting effect of mercury, and specifically thimerosal (TM - an ethyl mercury-containing preservative included in some vaccines administered to mothers and infants), on TH status. Surprisingly, no data on plasma TH levels following TM exposure have been reported and very few studies have explored the effect of methyl mercury (MetHg) on TH plasma levels. Studies in mice have shown that although the levels of TH in maternal and fetal plasma were not affected by short gestational exposure to MetHg, fetal brain deiodinase type 2 (D2) activity was increased (2). On the other hand, MetHg inhibited D2 activity both in neuroblastoma (3) and rat pituitary tumor cells *in vitro* (4). We have recently reported a decrease in cerebellar D2 activity following the perinatal TM exposure in SHR rats (5). Importantly, a majority of the active TH hormone in the brain is due to the activity of D2, a selenoenzyme that converts the pro-hormone thyroxine (T4) to the active hormone, 3',3,5-triiodothyronine (T3) (6); a relatively small proportion of brain T3 is transported from the plasma. Thus, it is possible that brain TH levels are altered by TM exposure, while plasma levels remain unchanged. Interestingly, T3 produced by D2 in the brain and T3 derived from the plasma are involved in the regulation of distinct gene subpopulations (7). Specifically, a deficiency of D2 results in the up-regulation of genes negatively regulated by TH (7). Thus, a decrease in D2 activity is likely to result in local hypothyroidism within the brain, and contribute to both TM and MetHg neurotoxicity through altered expression of specific subpopulation of TH-dependent genes negatively regulated by T3.

In the present study we examined this hypothesis, by assessing the effect of TM exposure on both positively and negatively regulated TH-dependent gene expression in the cerebellum. While recently (5), we reported on the effect of TM on two cerebellar genes - the Odf4 gene which was activated in males, and the cold inducible RNA binding protein (Cirbp) gene that was not affected by TM exposure (5) - present report includes data on nine additional genes that include both TH-dependent genes as well as other genes critical for cerebellar development. We report here for the first time up-regulation of a gene negatively regulated by T3

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Thimerosal stimulates focal adhesion kinase and cytoskeletal changes by redox modulation

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Abstract

Thimerosal is one of the most widely used preservatives and has been reported to cause chemically mediated side effects. However, the mechanism of the side effects is not clearly understood yet. In the present study, we showed that HeLa S cells treated by thimerosal generated reactive oxygen species (ROS). Thimerosal-generated ROS stimulated the tyrosine phosphorylation of focal adhesion kinase (FAK) and also induced cytoskeletal changes. Pretreatment with intracellular calcium chelator, BAPTA did not block the thimerosal-mediated FAK tyrosine phosphorylation. On the other hand, either FAK inhibitor, tyrphostin or ROS scavenger, *N*-acetyl-L-cysteine (NAC) suppressed the tyrosine phosphorylation and cytoskeletal changes. These results suggest that thimerosal seems to induce FAK tyrosine phosphorylation and cytoskeletal changes by ROS generation but not by intracellular calcium mobilization. We think the present finding can be an important clue to understanding the mechanism of thimerosal-mediated side effects, such as contact dermatitis, and allergy.

Keywords: Focal adhesion kinase; Tyrosine phosphorylation; Cytoskeletal change; Thimerosal; Reactive oxygen species

1. Introduction

The antibacterial and antifungal activity of thimerosal has been used as preservatives for various biological products, including vaccines, cleaning solutions for eye lenses, as well as cosmetics. The wide use of thimerosal in biological materials has often resulted in public health issues by causing thimerosal-mediated side effects such as contact dermatitis, and inflammatory responses [1,2]. Thimerosal in cleaning solutions for eye lenses has been previously shown to produce cytotoxicity for corneal epithelial cells [3–5]. The molecular mechanism of the side effects, however, has not been clearly described yet. The most well-characterized biological activity of thimerosal is the intracellular calcium mobilization by thimerosal, which has been previously manifested in many different cell types, such as smooth muscle cells, endothelial cells, HeLa cells, platelets, neutrophils, lymphocytes, and so on [6]. Thimerosal is a thiosalicylic acid derivative containing ethyl mercury (Fig. 1A) and the mercury atom is essential for the calcium mobilizing activity of the compound. Thiosalicylic acid, a thimerosal structural analogue, which does not contain the ethyl mercury, has no calcium releasing activity. Furthermore, the mercury atom of thimerosal gives the compound an oxidative character and some protein sulfhydryl groups, such as ATPase Ca^{2+} pump of the sarcoplasmic reticulum, are reported to be redox modulated by thimerosal [7]. In this study, we suggest that focal adhesion kinase (FAK) is one of the important target molecules of thimerosal.

FAK is a nonreceptor protein tyrosine kinase (PTK) and a key mediator of integrin signaling, which implicates its regulatory roles in cell adhesion, spreading, migration as well as cell survival and proliferation. Stimulation of FAK tyrosine phosphorylation has been reported in many different cell types by various kinds of stimuli, which can be integrin-independent or integrin-dependent [8]. Upon stimulation, FAK can be autophosphorylated on tyrosine 397, recruiting other nonreceptor PTKs, pp60src and pp59fyn, via their SH2 domains [9], which can create additional tyrosine phosphorylation on other residues of FAK. As one of the integrin-independent signals, H_2O_2 has been reported to induce FAK tyrosine phosphorylation in vascu-

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Dialysis
Transplantation

Preliminary Communication

Mercury exposure in protein A immunoadsorption

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Abstract

Background. Immunoadsorption is increasingly used to treat antibody-mediated autoimmune diseases. To prevent microbial growth during storage, reusable protein A–Sepharose gel columns are primed with ethyl mercury thiosalicylate (thiomersal, 0.1% solution) and rinsed with phosphate buffer before use. In this study, we tested the hypothesis of systemic mercury exposure in protein A immunoadsorption.

Methods. Whole blood mercury levels were measured by atomic absorption spectroscopy before and after protein A immunoadsorption (11 patients, 26 treatments), anti-IgG immunoadsorption (eight patients, 13 treatments) and LDL apheresis (DALI and Therasorb systems; nine patients, 14 treatments).

Results. Patients treated with protein A immunoadsorption had significantly elevated baseline mercury levels compared with the other groups, which were not different from healthy controls. Following protein A immunoadsorption, mercury levels increased from 5.9 ± 1.4 μg/l (mean ± SEM, normal, < 5 μg/l) to 32.3 ± 5.7 μg/l, $P < 0.001$). In one intensively treated patient, acute neurological toxicity developed at a mercury level of 107 μg/l. Symptoms abated slowly and did not recur after switching to a thiomersal-free system and chelation therapy. No mercury release to patients occurred in anti-IgG immunoadsorption or LDL apheresis treatments.

Conclusion. This preliminary report suggests that protein A immunoadsorption columns primed with thiomersal during storage may cause a sustained increase of systemic mercury concentrations, which exceed current safety recommendations in a proportion of patients. Considering the potential for mercury-induced toxicity, every effort should be undertaken to reduce systemic mercury exposure, either by adding chelators to the rinsing solution or ideally by replacement of thiomersal.

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Keywords: immunoadsorption; mercury; thiomersal; toxicity; tremor

Introduction

Immunoadsorption is a novel adsorption technique for semi-selective extracorporeal removal of circulating autoantibodies in disorders such as recurrent kidney graft rejection due to HLA hypersensitization [1], renal autoimmune disorders [2], haemophilia with inhibitors to factor VIII or IX [3], congestive heart failure [4] and neurological autoimmune diseases including myasthenia gravis and Guillain–Barre syndrome [5]. Current apheresis systems employ reusable columns designed for long-term storage (20–50 treatment sessions). To prevent microbial growth, staphylococcal protein A–Sepharose (PA) columns are primed with ethyl mercury thiosalicylic acid (buffered thiomersal 0.1% solution) during storage and rinsed with phosphate buffer before use [6]. In contrast, anti-IgG immunoadsorbers are stored after priming with phosphate-buffered saline (PBS)–sodium azide [7].

The long half-life of ethylmercury could theoretically result in accumulation and toxicity during chronic application, as discussed in the context of thiomersal-containing vaccines [8]. Following recommendations from the US Public Health Service and the American Academy of Pediatrics, thiomersal has been largely replaced in infant vaccines, although no clear evidence of potential health and development problems has been demonstrated so far [8]. Organic mercury toxicity affects mainly the central nervous system (CNS), with symptoms such as lethargy, loss of appetite, weight loss, tremor, memory loss, sleep disturbance, emotional lability and confusion [9]. Furthermore, mercury may cause significant damage to the haematopoietic and renal system [9]. Since PA immunoadsorption has been used successfully for several years at our department, the lack of long-term safety data led us to investigate the hypothesis of a thiomersal-related mercury release during apheresis.

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Reduced tubulin tyrosination as an early marker of mercury toxicity in differentiating N2a cells

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Abstract

The aims of this work were to compare the effects of methyl mercury chloride and thimerosal on neurite/process outgrowth and microtubule proteins in differentiating mouse N2a neuroblastoma and rat C6 glioma cells. Exposure for 4 h to sublethal concentrations of both compounds inhibited neurite outgrowth to a similar extent in both cells lines compared to controls. In the case of N2a cells, this inhibitory effect by both compounds was associated with a fall in the reactivity of western blots of cell extracts with monoclonal antibody T1A2, which recognises C-terminally tyrosinated α-tubulin. By contrast, reactivity with monoclonal antibody B512 (which recognises total α-tubulin) was unaffected at the same time point. These findings suggest that decreased tubulin tyrosination represents a neuron-specific early marker of mercury toxicity associated with impaired neurite outgrowth.

Keywords: Mercury; Thimerosal; Tubulin tyrosination; Neurite outgrowth

1. Introduction

Increased worldwide industrialisation has led to higher levels of pollution by potent neurotoxins such as methyl mercury. This compound has been linked with numerous toxic episodes in man, which were invariably associated with disturbed motor function and mental impairment, causing symptoms such as fever, tiredness, tremors and delusions in severe cases (Castoldi, 2001; Jacobson, 2001). Of particular concern is the fact that methyl mercury can cause congenital poisoning via transplacental transfer (Jacobson, 2001), accounting for some of the reported cases of infant poisoning and raising awareness of its developmental toxicity.

The organic form of mercury is considered to be more toxic than inorganic mercury, presumably due to differences in its uptake and chemical reactivity (O'Kusky, 1992). However, although widespread exposure to methyl mercury is rare nowadays, aquatic microorganisms can convert inorganic mercury into organic mercury, which may then be ingested by larger species and eventually work its way up the human food chain, affecting both adults and children (Atchison and Hare, 2004; Counter and Buchanan, 2004). There is also concern over the use of ethyl mercury thiosalicylate (thimerosal) as a preservative in certain vaccines and topical medications, some of which are administered to infants, as the very young are believed to be more sensitive to mercury toxicity (Goldman and Shannon, 2001). Thus, the risk of mercury toxicity remains a cause for concern in today's society.

It has been suggested from cell culture studies that the neurotoxicity of methyl mercury is linked to its ability to inhibit axon outgrowth and to disrupt microtubules in developing neurons (Graff et al., 1997; Miura et al., 1999; Heidemann et al., 2001; Parran et al., 2003). Indeed, subpopulations of dynamic microtubules enriched in C-terminally tyrosinated α-tubulin were shown to be more sensitive to disruption by methyl mercury (Graff et al., 1997). By

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Thimerosal induces oxidative stress in HeLa S epithelial cells

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Abstract

Thimerosal is one of the most widely used preservatives and is found in a variety of biological products, including vaccines, contact lens cleaning solutions, and cosmetics. It has been reported to have harmful effects on epithelial tissues, such as causing conjunctivitis or contact dermatitis. However, the molecular mechanism of its toxicity has not been characterized using epithelial tissues. In the present study, we report that reactive oxygen species play a key role in thimerosal-induced cytotoxicity in HeLa S epithelial cells. Thimerosal significantly reduced HeLa S cell viability and it was associated with a decrease in intracellular glutathione levels. Flow cytometric cell cycle analysis showed a marked increase in the hypodiploidic cell population, indicating apoptosis of thimerosal-treated cells. The apoptotic cell death of epithelial cells was confirmed by observing a significant increase of caspase-3 activity in the cytosolic fraction of the treated cells. Thimerosal also induced a concentration-dependent increase of genomic DNA fragmentation, a biochemical hallmark of apoptosis. Hoechst 33342 nuclear staining demonstrated apoptotic-fragmented multinuclei in thimerosal-treated cells. All the thimerosal-mediated toxic responses observed in the present study were almost completely suppressed by pretreating cells with *N*-acetyl-L-cysteine, a radical scavenger. Taken together, these results suggest for the first time that epithelial cytotoxicity of thimerosal is mediated by oxidative stress.

Keywords: Thimerosal; Epithelial toxicity; Reactive oxygen species; Glutathione; Caspase-3

1. Introduction

The antiseptic and antimicrobial activities of thimerosal have led to its use as a preservative in biological products, including vaccines, cleaning solutions for contact lenses, and cosmetics since the 1930s. However, there have been several reports that thimerosal has potential side effects, including causing inflammatory diseases (Clarkson, 2002; Lopez Bernal and Ubels, 1991). In particular, it has been speculated that thimerosal preservative in juvenile vaccines is a causative factor of autism in the vaccinated children (van't Veen, 2001); however, this idea is still the subject of much debate. Recently, the mechanisms of this potential side effect have been illustrated in neuronal cells (Ball et al., 2001; Geier and Geier, 2004; James et al., 2005) and T-lymphocytes (Lebrec et al., 1999; Makani et al., 2002). In a previous study, we have directly demonstrated for the first time that thimerosal can generate reactive oxygen species (ROS), including hydrogen peroxide in cultured mammalian cells (Kim et al., 2002a). The ROS generation stimulates focal adhesion kinase and cytoskeletal rearrangement, resulting in the typical morphological changes observed in thimerosal treatment. It appears to generate not only hydrogen peroxide but also other species of ROS (E. Kim, unpublished data). Apparently, thimerosal-induced intracellular calcium upregulation is also dependent on the generation of ROS by thimerosal (Kim et al., 2002b).

The side effects of thimerosal on epithelial tissues, such as conjunctivitis and contact dermatitis, have been previously reported (Garner, 2004; Belsito, 2002; Pratt et al., 2004; Wantke et al., 1994; Lebrec et al., 1999). However, the molecular mechanism of its toxicity has not been clearly elucidated in epithelial tissue. While the adverse effects of thimerosal on an epithe-

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REVIEW

Hypersensitivity Reactions to Vaccine Constituents: A Case Series and Review of the Literature

Jonathan S. Leventhal, BA, Emily M. Berger, MD, Jeremy A. Brauer, MD, and David E. Cohen, MD, MPH

Vaccines are composed of immunogens, preservatives, adjuvants, antibiotics, and manufacturing by-products. Components of vaccines may rarely elicit adverse reactions in susceptible individuals, thus raising concerns regarding vaccine safety. In this report, we add to the medical literature 3 cases of cutaneous delayed-type hypersensitivity to the vaccine preservative aluminum. We provide a review of major constituents in vaccines that have elicited immediate-type or delayed-type hypersensitivity reactions and describe their clinical manifestations. We include a table of the Food and Drug Administration–approved vaccines, which lists the quantities of major components including ovalbumin (egg protein), gelatin, aluminum, neomycin, 2-phenoxyethanol, thimerosal, and formaldehyde. Our goals were to inform physicians on the variety of hypersensitivity reactions to common vaccines and to provide information on the choice of vaccines in patients with suspected hypersensitivity.

SINCE THE invention of vaccines more than 200 years ago, once-fatal infectious diseases have become preventable on a global level. Vaccines consist of immunogens (bacterial or viral antigens) in addition to preservatives, adjuvants, antibiotics, and by-products or residuals from the manufacturing process.[1] The additional constituents of vaccines are important in their development, immunogenicity, and safety. Rare case reports document adverse reactions to specific components of vaccines. As such, with the increasing number of available and recommended vaccines, there are concerns about the potential toxicity associated with vaccines.[2,3]

Adverse reactions to vaccines include immediate-type (ie, immunoglobulin E mediated) and delayed-type (ie, type 4) hypersensitivity reactions. Fortunately, the rate of vaccine-induced adverse effects is low, ranging from 4.8 to 83 per 100,000 doses of the most frequently used vaccines.[4] Furthermore, life-threatening anaphylaxis remains extremely rare, averaging 1 per 1,500,000 doses.[4]

We report 3 cases of delayed-type hypersensitivity reactions to vaccine components and review constituents that may elicit adverse reactions. Whereas adverse reactions to vaccines are not limited to hypersensitivity reactions, this review will focus on this aspect of vaccine adverse effects. We discuss some constituents that can cause immediate-type hypersensitivity, but mainly review delayed-type hypersensitivity reactions. This is an updated report that reevaluates our previous review to: illustrate the problem through the 3 included cases; explore constituents that cause immediate-type hypersensitivity, delayed-type hypersensitivity, or both; and include a comprehensive table of the Food and Drug Administration (FDA)–approved vaccines, including the quantities of various vaccine components (ie, ovalbumin, gelatin, aluminum, neomycin, 2-phenoxyethanol [2-PE], thimerosal, and formaldehyde).[2]

CASE SERIES

Case 1

An 11-year-old girl presented 6 months after influenza vaccination and 14 months after a combined diphtheria, tetanus, and acellular pertussis (DTaP) vaccination for evaluation of pruritic eruptions after vaccine administration, partially relieved by oral antihistamines. See Table 1 for description of these reactions and patch test reactions. Past medical history was notable for von Willebrand disease, with no known allergy to eggs.

Case 2

A 5-year-old boy presented 2 months after DTaP vaccination for evaluation of subsequent exuberant cutaneous reaction that occurred 24 to 36 hours after immunization, relieved by oral prednisone. There was no known allergy to eggs. See Table 1 for description of these reactions and patch test reactions. Our institution's standard series of allergens for pediatric patients were not interpretable and obscured by a robust eczematous reaction.

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SHORT REPORT

Mercury intoxication presenting with tics

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Abstract

A 5 year old Chinese boy presented with recurrent oral ulceration followed by motor and vocal tics. The Chinese herbal spray he used for his mouth ulcers was found to have a high mercury content. His blood mercury concentration was raised. Isolated tics as the sole presentation of mercury intoxication has not previously been reported.

(*Arch Dis Child* 2000;**83**:174–175)

Keywords: tics; mercury poisoning; Chinese medicinal herb

Case report

A 5 year old Chinese boy of healthy unrelated parents presented to our hospital on two occasions—initially with oral ulceration, and then with motor and vocal tics. The oral ulceration, which mainly affected the left lateral aspect of his tongue, appeared approximately five weeks prior to the onset of tics. Herpetic ulceration was diagnosed and confirmed by the isolation of herpes simplex virus (HSV) type 1 from his tongue swab. The lesion improved after treatment with a five day course of oral acyclovir (200 mg five times daily), but relapsed a few days after finishing the course of medication. The family then consulted a local pharmacist who prescribed for the child a Chinese medicinal herb (CMH) mouth spray, named "Watermelon Frost". The spray was said to be useful in controlling pain and healing difficult mucosal wounds.

Over the following weeks, his mother noticed an improvement in his oral symptoms but commented that he had become irritable and had been clearing his throat frequently. A transient skin rash was also noticed on his trunk a few days before his second admission. On the day of admission, he developed a sudden onset of motor tics that consisted of eye blinking, head turning, and shoulder shrugging. There was no preceding history of flu like symptoms, head injury, or consumption of other drugs or herbs. His general health had been good and his developmental milestones were normal. There was no family history of any psychiatric or neurological problems. He had been on a normal unrestricted diet and there was no history of excessive seafood consumption.

He looked well on examination, which was interrupted by episodes of motor tics as described. Blood pressure was 110/65 mm Hg and heart rate 96 beats per minute. No skin rash or desquamation on the palms and soles were noted. There was a small healing ulcer at the tip of his tongue. His speech and gait were normal. Cardiovascular, respiratory, abdominal, and neurological examination did not reveal any abnormalities.

Initial investigations including complete blood count, renal function tests and electrolytes, liver enzymes, immunoglobulins, complement, as well as urine analysis and toxicology screen were all normal. Electroencephalography, cranial computerised tomography, and magnetic resonance imaging were also normal. Serum antineuronal antibody as determined by flow cytometry (less than 5 MIF units) and ASOT (less than 60 Todd units) were not raised.

On further questioning, our patient admitted that he had been using the CMH mouth spray up to 20 times a day for the preceding four weeks, when the recommended dose was only one spray twice a day. As the use of herbal medication always arouses the suspicion of heavy metal exposure in the locality, screening for heavy metals was performed.

The herbal spray was digested with concentrated nitric acid (12 mmol/l) for five days at room temperature, and total mercury concentration was then measured by cold vapour atomic absorption spectrophotometry (Flow Injection Mercury System, Perkin Elmer Corp., Norwalk, Connecticut, USA). Arsenic, manganese, and lead contents were determined by graphite furnace atomic absorption spectrophotometry (SIMAA 6000 Analyser, Perkin Elmer Corp.). The blood concentrations for lead and manganese were 0.31 μmol/l (normal <1.5 μmol/l) and 246 nmol/l (normal 70–280 nmol/l); urine arsenic was 10 nmol/mmol creatinine (normal <68 nmol/mmol). Blood mercury concentration was 83 nmol/l (normal for adults <50 nmol/l). The mercury content of the spray was 878 ppm (2% methylmercury and 98% inorganic mercury). There was also a significant difference in mercury content between different brands as well as batches of the same brand of CMH (see table 1). Sensory and motor nerve conduction velocities in our patient were normal. Detailed neuropsychological assessment was also normal.

The CMH spray was discontinued on admission. As the patient was clinically stable and his neurological symptoms improving,

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Thimerosal-Induced Apoptosis in Mouse C2C12 Myoblast Cells Occurs through Suppression of the PI3K/Akt/Survivin Pathway

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Abstract

Background: Thimerosal, a mercury-containing preservative, is one of the most widely used preservatives and found in a variety of biological products. Concerns over its possible toxicity have reemerged recently due to its use in vaccines. Thimerosal has also been reported to be markedly cytotoxic to neural tissue. However, little is known regarding thimerosal-induced toxicity in muscle tissue. Therefore, we investigated the cytotoxic effect of thimerosal and its possible mechanisms on mouse C2C12 myoblast cells.

Methodology/Principal Findings: The study showed that C2C12 myoblast cells underwent inhibition of proliferation and apoptosis after exposure to thimerosal (125–500 nM) for 24, 48 and 72 h. Thimerosal caused S phase arrest and induced apoptosis as assessed by flow cytometric analysis, Hoechst staining and immunoblotting. The data revealed that thimerosal could trigger the leakage of cytochrome c from mitochondria, followed by cleavage of caspase-9 and caspase-3, and that an inhibitor of caspase could suppress thimerosal-induced apoptosis. Thimerosal inhibited the phosphorylation of Akt^{ser473} and survivin expression. Wortmannin, a PI3K inhibitor, inhibited Akt activity and decreased survivin expression, resulting in increased thimerosal-induced apoptosis in C2C12 cells, while the activation of PI3K/Akt pathway by mIGF-I (50 ng/ml) increased the expression of survivin and attenuated apoptosis. Furthermore, the inhibition of survivin expression by siRNA enhanced thimerosal-induced cell apoptosis, while overexpression of survivin prevented thimerosal-induced apoptosis. Taken together, the data show that the PI3K/Akt/survivin pathway plays an important role in the thimerosal-induced apoptosis in C2C12 cells.

Conclusions/Significance: Our results suggest that in C2C12 myoblast cells, thimerosal induces S phase arrest and finally causes apoptosis via inhibition of PI3K/Akt/survivin signaling followed by activation of the mitochondrial apoptotic pathway.

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Introduction

Thimerosal is a water-soluble derivative of thiosalicylic acid. Due to its antimicrobial properties, it is widely used as a preservative in vaccines, ophthalmic products and cosmetics [1]. The safety of thimerosal has recently been questioned based on a number of studies that indicate to its possible risk of toxicity [2–5]. Thimerosal has been shown to cause a number of immunological and neurotoxic changes in microglia and astrocytes [1,6–9], and also been shown to induce apoptosis of SK-N-SH human neuroblastoma cells via the c-Jun N-terminal kinase pathway [10] and induce DNA breaks, caspase-3 activation, membrane damage and cell death in cultured human neurons and fibroblasts [11]. Woo et al. found that it could induce G_2/M phase arrest in human leukemia cells via the generation of reactive oxygen species and release of cytochrome C [12], while Makani et al. indicated that thimerosal could induce apoptosis in T cells via the mitochondrial pathway [13]. More recently, thimerosal has been classified as the second most common allergen after nickel [14–18], and also been shown to induce epithelial cytotoxicity via oxidative stress in HeLa S epithelial cells and apoptosis in human SCM1 gastric cancer cells via activation of the p38 MAP kinase and caspase-3 [19].

When people were vaccinated intramuscularly, thimerosal in vaccine directly contacts and might cause injury to skeletal muscle cells; this might be the reason for inflammation or amyotrophia at the injection site. However, little is known about the acute reactions of skeletal muscle tissues and cells following short-term exposure to thimerosal at nanomolar concentrations. Repair of degenerated muscles depends on a small group of skeletal muscle stem cells known as satellite cells [20]. Satellite cells form a group of quiescent muscle precursor cells that reside beneath the basal lamina and provide the predominant source of additional myonuclei for muscle growth [21,22]. Once activated, satellite cells give rise to myoblasts that proliferate, differentiate, and fuse

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Transcriptomic Analyses of Neurotoxic Effects in Mouse Brain After Intermittent Neonatal Administration of Thimerosal

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Thimerosal is a vaccine antimicrobial preservative which has long been suspected an iatrogenic factor possibly contributing to neurodevelopmental disorders including autism. The association between infant vaccine thimerosal exposure and autism remains an open question. Although thimerosal has been removed from mandatory childhood vaccines in the United States, thimerosal-preserved vaccines are still widely used outside of the United States especially in developing countries. Notably, thimerosal-containing vaccines are being given to the newborns within the first 12–24 h after birth in some countries. To examine the possible neurotoxic effects of early neonatal exposure to a higher level of thimerosal, FVB mice were subcutaneously injected with thimerosal-mercury at a dose which is 20× higher than that used for regular Chinese infant immunization during the first 4 months of life. Thimerosal-treated mice exhibited neural development delay, social interaction deficiency, and inclination of depression. Apparent neuropathological changes were also observed in adult mice neonatally treated with thimerosal. High-throughput RNA sequencing of autistic-behaved mice brains revealed the alternation of a number of canonical pathways involving neuronal development, neuronal synaptic function, and the dysregulation of endocrine system. Intriguingly, the elevation of anterior pituitary secreting hormones occurred exclusively in male but not in female thimerosal-treated mice, demonstrating for the first time the gender bias of thimerosal-mercury toxicity with regard to endocrine system. Our results indicate that higher dose of neonatal thimerosal-mercury (20× higher than that used in human) is capable of inducing long-lasting substantial dysregulation of neurodevelopment, synaptic function, and endocrine system, which could be the causal involvements of autistic-like behavior in mice.

Key words: **thimerosal; transcriptomic analyses; anterior pituitary; hormone; neurotoxicity; autistic disorder.**

Thimerosal (sodium ethylmercury thiosalicylate, 49.6% mercury (Hg) by weight) has been used as an antimicrobial preservative in many vaccines and medicinal preparations since 1930s (Pless and Risher, 2000). It rapidly metabolizes to ethylmercury and subsequently to inorganic mercury forms which accumulate in different organs/tissues including the brain for months or years (Qvarnstrom *et al.*, 2003). The neurotoxicity of ethylmercury has been well known (Zhang, 1984). Because the blood-brain barrier of newborns is not well-developed, and the developing brain is uniquely vulnerable to neurotoxic hazard exposure, thimerosal-mercurials are suspected pathogenic factors in the etiology of several neurodevelopmental disorders, including autism (Bernard *et al.*, 2001; Geier and Geier, 2003, 2005, 2006b; Hewitson *et al.*, 2010; Majewska *et al.*, 2010; Young *et al.*, 2008). However, the association between thimerosal exposure via childhood vaccinations and neurodevelopmental disorders such as autism remains an open question (Blaxill *et al.*, 2004; Kern *et al.*, 2012; Nelson and Bauman, 2003). Several independent epidemiological investigations support a hypothesis linking this disorder with postnatal exposure to mercurials (Gallagher and Goodman, 2010; Geier and Geier, 2003, 2004, 2006a,b; Mutter *et al.*, 2005; Young *et al.*, 2008), whereas the others do not support such a relationship (Heron and Golding, 2004; Hviid *et al.*, 2003; Immunization Safety Review Committee, 2004; Madsen *et al.*, 2003; Stehr-Green *et al.*, 2003; Thompson *et al.*, 2007; Verstraeten *et al.*, 2003). Nevertheless, due to concern of increased mercury exposure and elevated body burdens in children (Ball *et al.*, 2001), thimerosal has been removed from mandatory childhood vaccines in the United States (American Academy of Pediatrics and United States Public Health Service, 1999).

Thimerosal-preserved vaccines are still widely used outside of the United States especially in developing countries such as Brazil and China, where the advantages of multiuse vials of thimerosal-preserved vaccines take precedence over perceived mercury hazards (Dorea, 2007; WHO IRIS, 2002). According to 2001 United States vaccination schedule, each 1-year-old U.S. child could have been exposed to a total of 237.5 μg Hg from vaccines distributed at 2, 4, 6, and 12 months

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Toxicology

Toxicity of organic and inorganic mercury species in differentiated human neurons and human astrocytes

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ABSTRACT

Organic mercury (Hg) species exert their toxicity primarily in the central nervous system. The food relevant Hg species methylmercury (MeHg) has been frequently studied regarding its neurotoxic effects in vitro and in vivo. Neurotoxicity of thiomersal, which is used as a preservative in medical preparations, is to date less characterised. Due to dealkylation of organic Hg or oxidation of elemental Hg, inorganic Hg is present in the brain albeit these species are not able to readily cross the blood brain barrier. This study compared for the first time toxic effects of organic MeHg chloride (MeHgCl) and thiomersal as well as inorganic mercury chloride ($HgCl_2$) in differentiated human neurons (LUHMES) and human astrocytes (CCF-STTG1). The three Hg species differ in their degree and mechanism of toxicity in those two types of brain cells. Generally, neurons are more susceptible to Hg species induced cytotoxicity as compared to astrocytes. This might be due to the massive cellular mercury uptake in the differentiated neurons. The organic compounds exerted stronger cytotoxic effects as compared to inorganic $HgCl_2$. In contrast to $HgCl_2$ exposure, organic Hg compounds seem to induce the apoptotic cascade in neurons following low-level exposure. No indicators for apoptosis were identified for both inorganic and organic mercury species in astrocytes. Our studies clearly demonstrate species-specific toxic mechanisms. A mixed exposure towards all Hg species in the brain can be assumed. Thus, prospectively coexposure studies as well as cocultures of neurons and astrocytes could provide additional information in the investigation of Hg induced neurotoxicity.

1. Introduction

Organic mercury (Hg) compounds are important neurotoxicants capable of damaging the developing and adult nervous system [1]. Due to its accumulation in the aquatic food chain, chronic exposure to methylmercury (MeHg) via seafood intake still poses a risk to human health [2]. Ethylmercury (EtHg) containing thiomersal, used as a preservative in medical preparations including vaccines, is of particular concern since it has been linked to autism [3]. Although organic Hg compounds, especially methylmercury (MeHg), have been extensively studied, the mechanisms of Hg species mediated neurotoxicity remain not completely understood [4]. Inorganic Hg^{2+} does not readily cross the blood brain barrier. Probably therefore effects of inorganic Hg^{2+} species on brain cells are not well characterized [5]. Nevertheless, it should be noted that inorganic Hg is present in the brain due to dealkylation of organic species or an oxidation of elemental Hg, which originates e.g., from the outgassing of amalgam fillings [6,7].

In the literature only a few in vitro studies exist, either comparing effects of one Hg species, especially MeHg, in different brain associated cells or comparing different Hg species in one cell type. Sanfeliu et al. performed in vitro cytotoxicity studies in primary proliferating human astrocytes and neurons, indicating an enhanced sensitivity of neurons towards MeHg as compared to astrocytes [8]. In vitro studies in primary proliferating astrocytes and neurons from murine cerebella confirmed these results [9].

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Suppression by Thimerosal of *Ex-Vivo* CD4$^+$ T Cell Response to Influenza Vaccine and Induction of Apoptosis in Primary Memory T Cells

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Abstract

Thimerosal is a preservative used widely in vaccine formulations to prevent bacterial and fungal contamination in multidose vials of vaccine. Thimerosal was included in the multidose non-adjuvanted pandemic 2009 H1N1 vaccine Panenza. In the context of the analysis of the *ex-vivo* T cell responses directed against influenza vaccine, we discovered the *in vitro* toxicity Panenza, due to its content in thimerosal. Because thimerosal may skew the immune response to vaccines, we investigated in detail the *ex-vivo* effects of thimerosal on the fate and functions of T cells in response to TCR ligation. We report that *ex-vivo* exposure of quiescent or TCR-activated primary human T cells to thimerosal induced a dose-dependent apoptotic cell death associated with depolarization of mitochondrial membrane, generation of reactive oxygen species, cytochrome c release from the mitochondria and caspase-3 activation. Moreover, exposure to non-toxic concentrations of thimerosal induced cell cycle arrest in G0/G1 phase of TCR-activated T cells, and inhibition of the release of proinflammatory cytokines such as IFN gamma, IL-1 beta, TNF alpha, IL-2, as well as the chemokine MCP1. No shift towards Th2 or Th17 cells was detected. Overall these results underline the proapoptotic effect of thimerosal on primary human lymphocytes at concentrations 100 times less to those contained in the multidose vaccine, and they reveal the inhibitory effect of this preservative on T-cell proliferation and functions at nanomolar concentrations.

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Introduction

Thimerosal is a preservative used widely in vaccine formulations to prevent bacterial and fungal contamination in multidose vials of vaccine [1] [2]. Thimerosal, named also thiomersal or merthiolate in clinical studies, is an ethylmercury-containing pharmaceutical compound that contains 49.6% mercury by weight and metabolizes into ethylymercury (etHg) and thiosalicylate [3]. Thimerosal has served as a preservative in vaccines since 1930, but in the late 1990 concerns came as more thimerosal-containing vaccines were added to the recommended infant and child immunization schedule [4]. Research on the specific *in vivo* toxicity of low doses of etHg relevant to vaccines has only recently been performed [5] [6,7]. *In vitro*, thimerosal has been shown to cause a number of neurotoxic changes, including neuronal mitochondrial cell death characterized by the release of cytochrome c and apoptosis inducing factor from mitochondria to cytosol [8], DNA breaks and caspase 3 activation in cultured human neuronal cells [9], and mitochondrial-mediated apoptosis in a human neuroblastoma cell line following exposure to μM concentrations of thimerosal [10]. The deleterious effects of thimerosal were also reported on HeLa S epithelial cells, inducing an oxidative stress and cell death that were completely suppressed by pretreating the cells with N-acetyl-l-cysteine (NAC), a radical scavenger [11]. Thimerosal could also cause S phase arrest followed by mitochondrial apoptosis in murine myoblast cells that occurred via inhibition of the PI3K/Akt/survivin signaling pathway [12]. Surprisingly, little is known about the impact of thimerosal on immune cell functions. Trompezinski *et al.* found that it induced an oxidative stress in monocyte-derived dendritic cells [13], and Agrawal et al. reported that thimerosal could exercise a Th2 promoting effect through modulation of functions of dendritic cells [14]. At the T cell level, thimerosal was reported to induce caspase-dependent mitochondrial apoptosis in the human leukemic Jurkat T cells [15,16].

In the context of the clinical trial MICIVAX, designed to compare the efficacy and safety of influenza vaccine in patients with inflammatory bowel disease (IBD) receiving immunosuppressive therapy with patients not receiving immunosuppressants, we

RESEARCH PAPER

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Thimerosal compromises human dendritic cell maturation, IL-12 production, chemokine release, and T-helper polarization

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Keywords: thimerosal, dendritic cells, Th polarization, cytokines, chemokines

Thimerosal is a preservative used in multidose vials of vaccine formulations to prevent bacterial and fungal contamination. We recently reported that nanomolar concentrations of thimerosal induce cell cycle arrest of human T cells activated via the TCR and inhibition of proinflammatory cytokine production, thus interfering with T-cell functions. Given the essential role of dendritic cells (DCs) in T-cell polarization and vaccine immunity, we studied the influence of non-toxic concentrations of thimerosal on DC maturation and functions. Ex-vivo exposure of human monocyte-derived DCs to nanomolar concentrations of thimerosal prevented LPS-induced DC maturation, as evidenced by the inhibition of morphological changes and a decreased expression of the maturation markers CD86 and HLA-DR. In addition thimerosal dampened their proinflammatory response, in particular the production of the Th1 polarizing cytokine IL-12, as well as TNF-α and IL-6. DC-dependent T helper polarization was altered, leading to a decreased production of IFN-γ IP10 and GM-CSF and increased levels of IL-8, IL-9, and MIP-1α. Although multi-dose vials of vaccines containing thimerosal remain important for vaccine delivery, our results alert about the ex-vivo immunomodulatory effects of thimerosal on DCs, a key player for the induction of an adaptive response

Introduction

Adaptive immunity plays a crucial role in natural host defense against pathogens and tumors, and it is central to the long-term protective effect of vaccines. The innate immune system functions to direct the adaptive immune response, both through antigen presentation by dendritic cells and by providing the key signals for the differentiation of naive $CD4^+$ T cells into functionally distinct T helper cell (Th) subtypes.[1,2] DCs act as a sentinel population that constantly samples the tissue microenvironment and takes up microbial cells through toll-like receptors (TLRs).[3] TLRs can detect multiple pathogen-associated molecular patterns (PAMPs),[4] including LPS detected by TLR4, resulting in the activation of NF-κB that drives the production of many proinflammatory cytokines, including IL-1, IL-6, TNF-α, and IL-12.[5] TLR-induced IL-12 is the key differentiation factor for Th1 cells.[6] Upon DC maturation with LPS, chemokines such as MIP1-α, MCP1, and IP-10 are rapidly upregulated for recruitment and maintenance of DCs at the inflammatory site.[7] Furthermore, a recent report highlighted the importance of DC-derived IP-10 in the development of stable DC–$CD4^+$ Th cell interactions.[8] IP-10 binds to CXCR3 on Th cells and is required for optimal Th1 cell induction in the lymph node.[8]

Thimerosal is a preservative used in multidose vials of vaccine formulations to prevent bacterial and fungal contamination.[9,10] Thimerosal is an ethylmercury-containing pharmaceutical compound that contains 49.6% mercury by weight and metabolizes into ethylmercury (etHg) and thiosalicylate.[11] Thimerosal is known as a contact allergen, and caution has been urged regarding significant side effects in therapeutic agents[12] and in vaccines[13] with specific issues related to infant-CNS.[14,15] Thimerosal has been shown to cause a number of toxic changes in vitro, including neuronal mitochondrial cell death,[16,17,18] oxidative stress and apoptosis of HeLa S epithelial cells,[19] and S phase arrest and apoptosis via inhibition of the PI3K/Akt/survivin pathway on the murine C2C12 myoblast cells.[20] Because thimerosal is one of the best-known skin sensitizers, several studies have been performed on human myeloid dendritic cells, which play an essential role in the initiation of allergic contact dermatitis. DC activation and associated immune functions are subject to regulation by their redox environment and thimerosal was reported to induce ROS production in DCs,[21] associated with their activation, as monitored by CD86 and HLA-DR overexpression,[22,23] and the secretion of TNF-α and IL-8.[24] The link between thimerosal-induced oxidative stress and DC activation was also addressed by Goth et al. who reported that thimerosal altered IL-6 synthesis elicited by exogenous ATP,

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BEING ON THE TRACK OF THIMEROSAL

REVIEW

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The common preservative thimerosal is one of the most important organic mercury compounds human populations are exposed to. It has toxic effect on several cell lines, and it also induces programmed cell death in *in vitro* experiments. Association is suggested between application of thimerosal-containing vaccines and the occurrence of neurodevelopmental disorders, like autism. While specific recommendations were made to eliminate thimerosal from vaccines, consistent evidence is still lacking for an association of exposure and disease. Unfortunately, it is very hard to study the molecular background of complex human diseases directly; however, investigations on more simple model organisms may lead to a better understanding of thimerosal as a possible disease inducing factor.

Keywords: vaccine, neurodevelopmental disorder, autism, programmed cell death

Human exposure to organic mercury compounds

Mercury is considered to be one of the most toxic metals. Humans may be exposed to organic mercury compounds by inhalation, or via oral, or dermal routes. The effects of exposure to organic mercury are primarily neurologic [1], but other organ systems may also be involved, like gastrointestinal, respiratory, hepatic, immune, dermal and renal [2]. The exposure of billions of people occurs from seafood contamination, the methylmercury in fish and shellfish, mercury vapour from amalgam tooth fillings, and ethylmercury in the form of thimerosal (TMS) added as an antiseptic to widely used vaccines [3]. TMS is also common as preservative in cosmetics, ear and nasal drops, tincture of Merthiolate for minor in-

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Review on the Toxicity of Ethylmercury, Including its Presence as a Preservative in Biological and Pharmaceutical Products

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Key words: ethylmercury; thimerosal; kinetics; clinical studies; occupational exposure; dietary exposure; biological preparations; methylmercury.

INTRODUCTION

The interest in ethylmercury has been raised lately by a letter sent to *The Lancet* suggesting that ethylmercurythiosalicylate preservative (product names: Thimerosal, Thiomersal, Merthiolate) in hepatitis B immunoglobulin (HBIG) caused severe ethylmercury intoxication.[1] Most likely as a reverberation of this letter, the Public Health Service, US Department of Health and Human Services and the American Academy of Pediatrics published a joint statement on Thimerosal in vaccines.[2] As the nature of this statement has not required documentation on the toxicity of ethylmercury and the authors of the letter sent to *The Lancet* made the diagnosis without the prudent evaluation of published data, this survey attempts to fill the gap. It is not concerned with contact allergy[3] or with acrodynia (Pink disease). The only reported case of acrodynia was the result of regular injection of gamma-globulin with ethylmercury preservative to a 20-year-old man who had a history of chronic skin rash and sensitivity to sulfonamide drugs.[4]

KINETICS

Experiments in mice showed no difference between the distribution of ethylmercury chloride and ethylmercurythiosalicylate,[5] but after equivalent doses in mice[6] or rats[7] more mercury was in blood and less in brain after ethyl- than after methylmercury treatment. The passage of methylmercury across the blood–brain barrier is helped by an active transport mechanism[8] whereas passage of ethylmercury is hindered by its larger size and faster decomposition.

The clearance half-time of mercury from the whole body (including pelt) of rats was ca. 35 days for both ethylmercury[9] and methylmercury,[10] and mercury cleared from blood with 16 days half-time after the administration of ethyl- and methylmercury.[7] This suggests that in man the 50-day clearance half-time for total mercury after exposure to methylmercury[11] may be valid for ethylmercury. But change in blood mercury concentration indicated faster clearance in four infused patients[5] and slower clearance after occupational exposure.[12] In the following text extrapolation of blood mercury concentration to the end of exposure is based on a 50-day clearance half-time. These clearance half-times are not corrected to the toxicologically important loss of alkylmercury by the cleavage of the alkyl–mercury bond, although, compared with methylmercury 3 days after five daily doses, ethylmercury-treated rats had in blood about 13-fold, in brain 3,6-fold and in kidney 3-fold more inorganic mercury.[7] The difference in decomposition has an effect on the red blood cell to plasma mercury concentration ratio. Although this ratio was 7 in patients poisoned by dietary exposure to methylmercury[13] and 20 after a single oral dose[14] 2–3 weeks after the infusion of ethylmercury it ranged from 2 to 4.[5] Most importantly, when equimolar doses were given to rats, methylmercury caused widespread and ethylmercury only patchy damage in the cerebellar granular.[7] Thus, both kinetic and toxicological studies indicate that the relationships of dose and blood mercury concentration to the risk of intoxication established for methylmercury overestimates the risk of ethylmercury intoxication.

NEUROTOXICOLOGY

None of the signs of ethyl- and methylmercury intoxication are specific. Owing to three large epidemics (two in Minamata and Niagta, Japan, and one in Iraq), very much more is known on the clinical toxicology of methyl- rather than ethylmercury. Thus, in Minamata 100% of the victims had paraesthesia and constriction of the visual field whereas the frequencies of other signs decreased in the following order: ataxia, dysarthria, impaired hearing, tremor. In Niagata the order was similar but the frequencies of constricted visual field and dysarthria were lower and similar.[15] In Iraq the frequency of adverse effects increased when the blood mercury concentration exceeded 0.5 $\mu g\ ml^{-1}$. In the 0.5–1.0 $\mu g\ ml^{-1}$ range 42% of the patients had paraesthesia, 11% had ataxia and 21% had blurred or tunnel vision. The frequencyies of dysarthria or hearing defects were 5%. In the range of 3.0–5.0 $\mu g\ ml^{-1}$ 20%

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Principal component analysis and discrimination of variables associated with pre- and post-natal exposure to mercury

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Abstract

The variance of variables associated with neurodevelopment at 180 days, pre-natal variables (Hg in placenta, blood and hair) and post-natal Hg exposure (including Thimerosal-containing vaccines, TCV) were examined in 82 exclusively breastfed infants using principal component analysis (PCA). This multivariate method was applied to identify hierarchy and sets of interrelated variables. The PCA yielded a two-factor solution, explaining 92% of variance and summarizing most of the relevant information in the dataset matrix: the first component represented birth weight and vaccine (first doses of Hepatitis B and DTP) variability and explained 57% of variance; the second component represented a gradient of neurodevelopment (Gesell scores) and explained 35% of variance. The third component explained only 3% of the remaining 8% variance. Beside CNS priming by breastfeeding, infant development (birth weight) and time of immunization with TCV should be considered in epidemiological studies. PCA can classify sets of variables related to vaccination and neuromotor development schedules, clearly discriminating between earlier and later TCV exposures of exclusively breastfed infants. In conclusion, the incommensurable concept of the chance of toxic risk caused by TCV-EtHg exposure against the proven benefit of immunization is in no way disputed here. However, infant neurodevelopmental (ND) disorders linked to Thimerosal-Hg stands in need of proof, but PCA points to the possibility of identifying exposure risk variables associated with ND schedules.

Keywords: Thimerosal; Ethylmercury; Methylmercury; Vaccines; Breastfeeding; Neurodevelopment; Infants

Introduction

Mercury's most widely recognized effects are neurological; the developing central nervous system (CNS) of fetus, infants and young children are vulnerable to these effects. Fetal exposure to methylmercury (MeHg, from fish consumption) is thought to lower intelligence and alter behavior. The Harvard Center for Risk Analysis panel quantified the impact of chronic MeHg exposure on cognitive development (Cohen et al., 2005) and concluded that pre-natal MeHg exposure sufficient to increase maternal hair-Hg by 1 μg/g at parturition decreases intelligence quotient by 0.7 points (Cohen et al., 2005). However, neurological effects of post-natal

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Prenatal and Postnatal Mercury Exposure, Breastfeeding and Neurodevelopment During the First 5 Years

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Objective: We evaluated the association between infant hair-Hg and Gesell schedules (GS).

Background: Longitudinal assessment of prenatal and postnatal Hg exposure during the first 60 months.

Methods: We used hair-Hg as a marker of postnatal Hg exposure (inorganic and methyl-Hg from breast milk, and ethyl-Hg from thimerosal) and GS measured at 6, 36, and 60 months.

Results: Hair-Hg at 6 months responded to events related to Hg exposure and breastfeeding. However, most neurodevelopment delays observed at 6 months were overcome with infant growth; at 60 months 87% of children showed adequate GS (> 85). Length of lactation and hair-Hg were each significantly correlated with GS, but in opposite ways: length of lactation was positive and significantly correlated with all GS at 60 months; hair-Hg concentrations were negative and significantly correlated with GS at 6 months ($r = -0.333$; $P = 0.002$) and 60 months ($r = -0.803$; $P = 0.010$), but not at 36 months. Multiple regression models showed that the GS outcome at 60 months depended on GS at 36 months that in turn was influenced by infants' developmental and Hg exposure variables. GS at 6 months was significantly influenced by prenatal (maternal and infant hair-Hg at birth) and postnatal Hg exposure at 6 months.

Conclusions: Until there is more refined approach to recognize children sensitive to Hg exposure, and in situations of uncertainty (EtHg exposure), the neurodevelopment benefit of breastfeeding should be recommended.

Key Words: thimerosal, ethylmercury, breastfeeding, neurodevelopment, Gesell scores, vaccines

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Mercury is neurotoxic and has been widely recognized as harmful to the developing brain; therefore, preventing exposure during prenatal and postnatal critical periods of central nervous system (CNS) development has been the object of public health policies. Fish advisories aim to restrict its consumption during pregnancy[1] whereas thimerosal-containing vaccines (TCVs) have been banned in rich countries[2] despite the WHO recognition that TCVs are safe to use in infants and children. Collectively, the skewed rationale and perceived double standards that underlie thinking of infant Hg exposure (from maternal sources) and safety of thimerosal in vaccines has spawned skepticism among stakeholders and uncertainties among health professionals.[3]

Despite epidemiologic studies showing no association between TCVs and autism, there are grounds for concerns about other neurodevelopment (ND) disorders. The ability of the newborn to handle Hg is modulated by the degree of immaturity that is, limited bile production and renal function, and underdeveloped metabolic pathways[3]; these impairments are further exacerbated by the various factors associated with Hg exposure: source (Hg chemical forms—inorganic and organic Hg), route (enteral breast milk vs parenteral TCVs) and pathway (intrinsic in breast-milk protein matrix vs extrinsic in pure chemical form-EtHg). Additionally, genotypes with decreased glutathione availability for Hg conjugation have been found to affect Hg metabolism.[3]

Newborn babies and infants (especially those from less developed countries) represent populations of greatest concern to ND adversities: those most sensitive (because of anatomical, physiologic, and biochemical immaturity) and those most exposed (when immunized with TCVs and after maternal fish consumption) to Hg.[4] Owing to great variation in infants' susceptibility and modifiable circumstances (anatomical and functional development), there is a recognizable risk that mild and transient ND disorders can occur as a result of TCVs. However, because subtle effects of Hg on the CNS may manifest themselves long after TCVs exposure, and as this drawback is coupled with lack of proper markers (of exposure and effects), there are serious difficulties in establishing cause and effects (or even association), especially at early stages of development. Therefore, ND associated with time differences of perinatal TCVs inoculation schemes has not been detected in exclusively breastfed infants[5]; as a result, it is important to differentiate low-risk from high-risk situations.

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REGULAR ARTICLE

Thimerosal exposure (from tetanus-diphtheria vaccine) during pregnancy and neurodevelopment of breastfed infants at 6 months

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Abstract

Aim: We studied the effect on neurodevelopment of infants who are exposed to thimerosal in tetanus-diphtheria (Td) vaccines during pregnancy.

Methods: We compared Gesell Developmental Schedules (GDS) of exclusive breastfed infants at 6 months born to mothers who received Td (1 to 3 doses) against those who were born to mothers who did not take such vaccines.

Results: Compared with the group of infants not exposed to ethylmercury in utero, the infants of exposed mothers showed no significant difference in neurodevelopment delays. Although there was a significant correlation between hair-Hg of mothers and hair-Hg of neonates (Spearman $r = 0.353$; $p = 0.0011$), there was no significant correlation between the level of in utero exposure to ethylmercury in Td vaccines and neonate's hair-Hg concentrations (Spearman $r = 0.060$; $p = 0.5922$). However, regression analysis showed that GDS at 6 months was significantly associated with total mercury concentration of neonate's hair but was not sensitive to the number of vaccines taken by the mother.

Conclusion: Early neurodevelopment of exclusively breastfed infants is sensitive to in utero exposure to mercury, but maternal thimerosal exposure in tetanus-diphtheria vaccines per se cannot portend clinical neurodevelopment delays measured by GDS at 6 months.

INTRODUCTION

Prenatal exposure to neurotoxic substances can cause morphological and functional anomalies in infants; Hg is one such substance. Long-term risk of neurological conditions or diseases can be the result of adverse responses to in utero exposures to environmental chemicals (arising from occupational hazards, drugs and tobacco smoking). Depending on the exposure, these chemicals can affect the central nervous system (CNS) leading to neurodevelopmental disabilities or can cause subtle changes capable of inducing adaptive responses (1); some of these responses are only noticeable through changes in behaviour in later years. Better protection of the CNS during the most vulnerable time includes avoiding exposure or remedying some of these early effects through breastfeeding (2).

Despite the universal use of small amounts of thimerosal as a preservative of vaccines since the 1930s, research on its toxicokinetics and toxicodynamics in neonates and infants is rare and very limited; and, as a consequence, knowledge of its Hg metabolite (ethylmercury – etHg) is derived mostly from methylmercury (meHg) studies. The mechanistic understanding of organomercurials' neurotoxicity is unquestionable, and etHg is no exception. However, our ability to understand the safety of small quantities of etHg derived from thimerosal-containing vaccines (TCV) is still unsatisfactory (3); its limited toxicological understanding is the result of studies in animals and theoretical models (4). Although there is no proven causation of permanent neurological disorders in children exposed to TCV-etHg, its plausibility has been inferred from neurotoxic disasters caused by accidental exposure to high levels of organic Hg compounds (5).

The infant brain takes unusually a long period to form, with some higher functions being sensitive to initial anatomical and physiological conditions. Thus, the vulnerability of the developing brain to neurotoxic substances is amply recognized (6). Organic Hg compounds are among the neurotoxic substances whose effects depend on the CNS structure at time of exposure (6). Therefore, the use of TCV during pregnancy has been cautioned (3,7,8). Schilthuis and van Wijnen (9) raised an alert about the risk of thimerosal-containing gammaglobulin preparations for the prevention of hepatitis A in pregnant women, recommending alternatives without thimerosal. However, in the case of the tetanus vaccine, it has never been questioned (10). Tetanus vaccines are strategically used to increase protection of mothers and neonates and are considered safe. However,

Abbreviations
CNS, vulnerable central nervous system; etHg, ethylmercury; GDS, Gesell Developmental Schedules; meHg, methylmercury; TCV, thimerosal-containing vaccines; Td, tetanus and diphtheria vaccine.

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Perinatal multiple exposure to neurotoxic (lead, methylmercury, ethylmercury, and aluminum) substances and neurodevelopment at six and 24 months of age

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ABSTRACT

We studied neurodevelopment in infants from two communities. Children living in the vicinity of tin-ore kilns and smelters – TOKS; $n = 51$) were compared to children from a fishing village (Itapuã; $n = 45$). Mean hair-Hg (HHg) concentrations were significantly higher in Itapuã children which received significantly ($p = 0.0000001$) less mean ethylmercury (88.6 μg) from Thimerosal-containing vaccines (TCV) than the TOKS children (120 μg). Breast-milk Pb concentrations were significantly higher in the TOKS mothers ($p = 0.000017$; 10.04 *vs.* 3.9 μg L^{-1}). Bayley mental development index (MDI) and psychomotor development index (PDI) were statistically significant (respectively $p < 0.0000001$, $p = 0.000007$) lower for the TOKS children only at 24 months of age. Multivariate regression analysis showed that MDI was negatively affected by breast-milk Pb and by HHg. PDI was positively affected by breastfeeding and negatively affected by ethylmercury. Milestone achievements were negatively affected by breast-milk Pb (age of walking) and by HHg (age of talking).

1. Introduction

Early life neurodevelopmental challenges and resulting disabilities due to cumulative exposure to hazardous substances begin at pregnancy and/or during the post-natal period. Additionally, socio-economic disparities associated with psychological stimuli can modulate trajectories that influence mental and psychomotor outcomes. Exposure to environmental neurotoxic substances, *per se* or in combination, can burden the central nervous system (CNS) of the fetus and young child.

Due to the increased pollution or environmental contamination, children nowadays are exposed to more man-made toxic agents than in the past (Landrigan et al., 2005). The number of toxic molecules that are introduced with modern-day manufactured goods (including biocides) has increased considerably. As a result of CNS immaturity, the unborn fetus and infant have to deal with different kinds of toxic substances co-occurring from multiple sources. Neurotoxic metals (*e.g.* lead, mercury, and aluminum) *per se* are known to negatively affect neurodevelopment even at low doses. Indeed, developmental effects have been demonstrated in animal models and have also been observed in children (Rice and Barone, 2000; Carpenter, 2001; Fox et al., 2012). As reviewed elsewhere (Rice and Barone, 2000; Fox et al., 2012), the effects of such substances can be developmental delays, transient or persistent neurological deficits, with neurobehavioral consequences in the individual and societal costs (Bellinger, 2004; Attina and Trasande, 2013). Worldwide, with the increase in manufactured goods and economic globalization, there is a high prevalence of exposure to neurotoxic chemicals *per se* or in combination.

Organic Hg compounds (methylmercury – MeHg, ethylmercury – EtHg) are comparably toxic and hazardous with demonstrable risks shown in animal and human studies (Dórea et al., 2013). While MeHg exposure is mainly through consumption of fish and seafood, EtHg exposure occurs only through Thimerosal-containing vaccines (TCV) widely used in pediatric populations of third-world countries. Additionally, besides EtHg, TCV contains adjuvant-Al (Dórea and Marques, 2010); individually, these substances are below the currently assumed toxicological threshold. However, cumulative

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Neurodevelopment of Amazonian children exposed to ethylmercury (from Thimerosal in vaccines) and methylmercury (from fish)

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ABSTRACT

Few studies have addressed co-occurring methylmercury (MeHg) from maternal origin and ethylmercury (EtHg) from Thimerosal-containing vaccines (TCVs) during infant's neurodevelopment. We studied children ($n=1139$) from the Western Amazon based on combined (low, intermediate, and high) exposure to chronic MeHg from fish consumption and acute TCV- EtHg. Neurodevelopment outcomes were age of walking and age of talking, and the Bayley Scale of Infant Development (BSID). The Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) were measured at six and 24 months of age. Median hair-Hg (HHg) at birth was 6.4 $\mu g\, g^{-1}$ in mothers, and 1.94 $\mu g\, g^{-1}$ in newborns; total (pregnancy and infancy) EtHg exposure ranged from 0 to 187.5 μg. The combined (MeHg + EtHg) exposure showed significant differences for MDI but not for PDI; however, there was a significant decrease in both MDI and PDI scores at 24 months. The increase in BSID delays (scores < 80) between six and 24 months was not discernible with regards to EtHg or MeHg exposure. We found a statistically significant increase in neurodevelopmental (BSID) delays related to the combined exposure to Hg (MeHg > EtHg). Neurodevelopment delays due to low-doses of organic mercury (albeit undiscernible) are not predictable but can be avoided by choosing low-Hg fish and providing Thimerosal-free vaccines.

1. Introduction

During developmental periods extending from prenatal stages, the brain is more vulnerable to the adverse effects of toxic insults than at more mature stages; however, experience-guided development drives neuro-cognition achievements. Early brain susceptibility to mercury toxicity (Clarkson et al., 2003) and adverse neurological outcomes have been reported in animal experiments (*in vivo* and *in vitro*) as well as in human epidemiological studies (Dórea, 2013; Grandjean et al., 2014). In fish-eating populations of the Amazon, during prenatal and postnatal life, not only methylmercury (MeHg) but also ethylmercury (EtHg) in Thimerosal-containing vaccines (TCV) are the main forms of organic mercury exposure to the developing human brain (Marques et al., 2013a).

The first indication of EtHg neurotoxic effects on development resulted from high doses during the accidental mass poisoning in Iraq (Clarkson et al., 1976). Children born to women exposed to food contaminated with organic Hg (both MeHg and EtHg) showed impairment derived from neurological examination scores and milestone (age of first walking and first talking) delays (Marsh et al., 1987). Despite strong evidence of potential effects of low-doses of Thimerosal/EtHg (Geier et al., 2015), studies addressing only TCV-EtHg exposures and association with neurodevelopmental effects are conflicting in population studies conducted in developed countries (Dórea, 2010).

Despite a shorter residence time in the blood, compared with MeHg, Thimerosal-EtHg stays longer in the brain of monkeys (Burbacher et al., 2005). Thimerosal/EtHg toxicity tests conducted *in vitro* (molecular and cellular level) have shown perturbations of toxicity pathways of equal magnitude to that found for MeHg (Dórea, 2013). Experimental studies with Thimerosal/EtHg doses (simulating TCV) on tissue structure, function, and animal behavior have demonstrated neurotoxic effects in different species such as monkeys, hamsters, mice, and rats (Dórea, 2013); untoward effects of Thimerosal-EtHg on neurodevelopment have also been found in population studies (Dórea, 2013; Geier et al., 2015).

During pregnancy and lactation, subsistence fish-eating Amazonian mothers can pass relatively large amounts of Hg to fetuses and to breastfed infants (Marques et al., 2013a; Vieira et al., 2013).

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Mercury toxicity (acrodynia) induced by long-term injection of gammaglobulin

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MOST COMMERCIALLY AVAILABLE gammaglobulin preparations contain merthiolate (sodium ethylmercurithiosalicylate), a mercury-containing compound, which serves as a bacteriostatic agent. Thus, patients receiving regular injections of gammaglobulin are potentially at risk for the development of mercury toxicity.

The signs and symptoms of mercury toxicity are varied and depend upon the form of the mercury compound, the length of time of exposure, and individual patient sensitivity.[1] Acrodynia or pink disease is a sensitivity reaction to mercury salts and is characterized by pink, scaling palms and soles, flushed cheeks, pruritus, photophobia, profuse sweating, irritability, and insomnia.[2] We report a patient with congenital agammaglobulinemia and receiving gammaglobulin, who developed signs and symptoms of acrodynia.

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CASE REPORT

This 20-year-old male with congenital agammaglobulinemia has been receiving gammaglobulin injections (Connaught Laboratories, Toronto, Canada) for approximately 15 years. Although no detectable levels of serum IgA, IgM, or IgE have been demonstrated while on replacement therapy, he has a history of sensitivity reactions involving a rash when exposed to sulfonamide drugs, two episodes of alopecia totalis, rheumatoid-like arthritis, iritis, and a chronic skin rash of undefined nature. He did not have any unusual eating habits, nor was there a history of ingestion of large quantities of fish or other foods known to be a source of mercury. Over the course of the last six months he developed pink, scaling, pruritic palms and soles, flushed cheeks, photophobia, irritability, a fine tremor, and altered sensation in his fingertips. The following investigations were normal or negative: erythrocyte sedimentation rate, white cell count and differential, alkaline phosphatase, SGOT, creatinine phospokinase, viral cultures, cryoglobulins, cold agglutinins, latex fixation, antinuclear factor, protoporphyrins, total hemolytic complement, C3, and C4. He was found to have a slowed nerve conduction velocity.

0022-3476/80/070153+03$00.30/0

Mercury Poisoning in Child Treated with Aqueous Merthiolate®

[Thimerosal Topical Solution, U.S.P. (1:1000)]

The Ohio Board of Pharmacy has received an investigative report from the Ohio Department of Health's Division of Epidemiology regarding the death of a 21-month old child due to mercury poisoning. The investigation strongly implicated the Thimerosal solution as "the source of mercury that subsequently resulted in the child's death" since no other source could be identified. The poisoning apparently resulted from the improper use of Thimerosal Topical Solution, U.S.P. (1:1000), prescribed by a physician for the treatment of chronic otitis media.

The investigation disclosed that the child had a history of recurrent otitis media and in July 1981 had polyethylene ventilation tubes placed in both ears. In February 1982 the child was diagnosed as having otitis media in the left ear and was placed on antibiotics. A few days later, a prescription was issued for one pint of Merthiolate® solution (1:1000) with one refill. The prescription sig. indicated the ear was to be irrigated with one ounce of the solution on a daily basis.

The irrigations continued for approximately eight days and were then increased to twice-a-day for approximately four weeks. During this time period, two pints of the Thimerosal solution were obtained by prescription and one pint purchased over-the-counter. Approximately two and one-half pints were used over a five-week period to irrigate the left ear of the child.

According to the investigative report, the parents were instructed to irrigate the ear by placing the syringe into the polyethylene ventilation tube and pumping the Merthiolate® solution through the tympanic membrane into the inner ear. This method resulted in the child ingesting the approximately two and one-half pints of the mercury-containing product over the five-week period.

While the use of Thimerosal solution (1:1000) for the treatment of otitis media appears to be a common practice only in the Central Ohio area, the Board of Pharmacy is issuing this Advisory regarding the use and sale of Thimerosal Topical Solution (1:1000). Thimerosal Topical Solution, U.S.P. (1:1000) is a stable, saline, borate-buffered solution of an organic-mercury compound, is indicated for use as a topical antiseptic, and is available as an over-the-counter drug product.

In January 1982 the federal Food and Drug Administration *proposed* a rule classifying Thimerosal, and all other over-the-counter mercury-containing drug products for topical antimicrobial use, as not generally recognized as safe and effective and as being misbranded [47 FR 436]. As of April 20, 1983, the rule has not been adopted.

The proposed rule by F.D.A. was based on the "Advisory Review Panel on OTC Miscellaneous External Drug Products" recommendations concerning OTC mercury-containing drug products. The Panel concluded that "Thimerosol is not safe for OTC topical use because of its potential for cell damage if applied to broken skin and its allergy potential. It is not effective as a topical antimicrobial because it's bacteriostatic action can be reversed."

Studies conducted by physicians at Children's Hospital in Columbus, Ohio, however, indicate that Thimerosal solution (1:1000) is effective in the treatment of otitis media and is safe if used correctly. Correct use involves "'flushing' the ear with the solution with the head tilted and, after a few seconds, tilt the head in the opposite direction to let the ear drain." It is also recommended that treatment with Thimerosal solution should not continue beyond a period of two weeks.

•

The Thimerosal solution should *not* be forceably introduced through drainage tubes into the middle ear. Precautions should be taken when using Thimerosal solution (1:1000). Whenever there is a potential for ingestion or absorption from broken or abraded skin or mucous membranes. Pharmacists should alert patients regarding the proper use of the product and point out the cautions appearing on the label, especially where the product has been prescribed or is being purchased for use on an infant. Accordingly, the product should not be available for purchase without the advice of a pharmacist and cautions concerning its proper use. □

Minutes of the Annual Meeting and Committee Reports will be in the August *Journal*

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Responsiveness of human monocyte-derived dendritic cells to thimerosal and mercury derivatives

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ABSTRACT

Several cases of skin sensitization have been reported following the application of thimerosal, which is composed of ethyl mercury and thiosalicylic acid (TSA). However, few in vitro studies have been carried out on human dendritic cells (DCs) which play an essential role in the initiation of allergic contact dermatitis. The aim of the present study was to identify the effect of thimerosal and other mercury compounds on human DCs. To address this purpose, DCs derived from monocytes (mono-DCs) were used. Data show that thimerosal and mercury derivatives induced DC activation, as monitored by CD86 and HLA-DR overexpression associated with the secretion of tumor necrosis factor α and interleukin 8, similarly to lipopolysaccharide and the sensitizers, 1-chloro-2,4-dinitrobenzene (DNCB) and nickel sulfate, which were used as positive controls. In contrast, TSA, the non-mercury part of thimerosal, as well as dichloronitrobenzene, a DNCB negative control, and the irritant, sodium dodecyl sulfate, had no effect. Moreover, oxidative stress, monitored by ROS induction and depolarization of the mitochondrial membrane potential, was induced by thimerosal and mercury compounds, as well as DNCB, in comparison with hydrogen peroxide, used as a positive control. The role of thiol oxidation in the initiation of mono-DC activation was confirmed by a pre-treatment with *N*-acetyl-L-cysteine which strongly decreased chemical-induced CD86 overexpression.
These data are in agreement with several clinical observations of the high relevance of thimerosal in patch-test reactions and prove that human mono-DCs are useful in vitro tools for determining the allergenic potency of chemicals.

Introduction

Thimerosal, which is also called merthiolate or thiomersal in clinical assays, is an organomercury product composed of ethyl mercury (EtHgCl) and thiosalicylic acid (TSA). Due to its antimicrobial properties, thimerosal is frequently found in topical antiseptic solutions and ointments. It is widely used as a preservative in vaccines, ophthalmic products and cosmetics (for a review, see Geier et al., 2007). Since the last decade, many cases of cutaneous reactions and skin sensitization in atopic children have been reported (Zenarola et al., 1995; Goncalo et al., 1996; Patrizi et al., 1999; Suneja and Belsito, 2001). Recently, statistical overviews of long-term trends in patch-test reactions in several countries reported large patch-test studies that reflected the high reactivity of thimerosal which was classified as the second most common allergen after nickel (Goon and Goh, 2006; Wattanakrai and Rajatanavin, 2007; Tudela et al., 2008; Cheng et al., 2008; Hammonds et al., 2009). However, the clinical relevance of thimerosal is still under discussion (Belsito, 2002; Slodownik and Ingber, 2005; Breithaupt and Jacob, 2008) and is currently pertinent as thimerosal is still added in several types of vaccines such as influenza A(H1N1), diphtheria toxoid, acellular pertussis and tetanus toxoid (www.afssaps.fr; www.eurosurveillance.org; www.fda.gov; www.vaccinesafety.edu). However, only few investigations were targeted on thimerosal effect towards the immune system. The effect of thimerosal has been studied mostly in rodents where a toxic reaction and some cases of autoimmunity were reported (Havarinasab et al., 2004; Silbergeld et al., 2005). Investigations on Jurkat T cell line have shown that thimerosal was able to induce apoptosis (Makani et al., 2002) and confirmed the sensitivity of T cells to thimerosal (Lee-Wong et al., 2005). Only recent studies (Goth et al., 2006; Agrawal et al., 2007) have demonstrated a direct effect of thimerosal on human dendritic cells (DCs), which play an essential role in the immune response and in the initiation of allergic contact dermatitis.

Several studies reported that immature DCs are generated in vitro from human monocytes (mono-DCs) and acquire a mature phenotype after 48 h exposure to sensitizers as previously described for 1-chloro-2,4-dinitrobenzene (DNCB) and nickel sulfide ($NiSO_4$) whereas non-sensitizers such as SDS had no significant effect (Aiba et al., 1997; Coutant et al., 1999; Guironnet et al., 2000; Tuschl et al., 2000; Hulette

Abbreviations: DCs, dendritic cells; mono-DCs, DCs derived from monocytes; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; TSA, thiosalicylic acid.

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Thimerosal, micromercurialism and chronic fatigue syndrome

Dear Sir

''The heightened susceptibility of the developing nervous system to mercury is well established, but little is known about factors that modulate sensitivity to repeated low-dose exposures delivered i.m. (*and*) or restricted to postnatal life'' [1], therefore, the following hypotheses are presented in hopes of generating significant thought and discussion supported by sound scientific method.

Chronic fatigue syndrome (CFS) and related illnesses are potentially caused by subacute mercury poisoning or ''micromercurialism'' [2]. It is also hypothesized that symptoms identified in both CFS and ''micromercurialism'' are present in a subgroup of adults of Anglo-descent who have received thimerosal-containing vaccines. Specifically, Westphel et al. [3] have reported that a ''Thimerosal allergic'' exhibits ''homozygous gene deletions of the glutathione-S-transferases M1 and T1''. Further as recently published, ''immune response genes may be linked to heritable factors mediating toxin-induced CNS damage'' [1].

If subacute mercury poisoning or ''micromercurialism'' is the cause of CFS, FMS and GWS then a drug compound containing one or more sulfhydryl (–SH) groups, known for their exceptional ability to chelate mercury, should promote improvement in the symptoms of patients suffering from the above-mentioned illnesses. The recovery rate reported for CFS patients is only 12%.

In an ongoing study involving the administration of glutathione, a tripeptide necessary for the body's general wellness, CFS (n = 478) patients were treated with glutathione and a glutathione · ATP (GSH · ATP) complex. Results of the study showed that patients receiving 200 mg GSH · ATP weekly exhibited an increase in natural killer (NK) cell count and relief from the chronic low grade temperature usually experienced by CFS patients. Patients receiving 300 mg GSH · ATP weekly reported an 84% improvement in symptoms [4].

Glutathione is required to transport heavy metals out of cells and/or across the blood–brain barrier into the extracellular fluid. The chelate, however, undergoes multiple hydrolysis steps as it makes its way through the liver, gall bladder and into the bowel. The hydrolyzed chelate is then reabsorbed as a L-cysteine complex. A steady-state enterohepatic circulation mechanism explains the aforementioned study results and further suggests that a secondary chelant is needed to shift the steady-state mechanism toward excretion. This hypothesis is supported in part by a recent paper presented at the seventh international AACFS conference [5].

A study of heritable factors in CFS patients is planned as well as a controlled study involving sulfhydryl-based chelants in concert with GSH · ATP therapy. The results will be presented early next year.

Sincerely,
Richard F. Miller, Sc.D.

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Effects of lipopolysaccharide and chelator on mercury content in the cerebrum of thimerosal-administered mice

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Abstract

Thimerosal is one of the best-known preservative agents for vaccines in the world but a relationship between its use and autism has long been suspected so that its effects on the brain need more detailed research. We here examined the influence of lipopolysaccharide injury to the blood–brain barrier on the penetration of mercury from thimerosal into mouse cerebrums, as well as the effect of chelator of heavy metals on cerebrum mercury content. Mercury can be expected to be detected in the cerebrum of normal mice, because the metal is present in standard mouse chow. When 60 μg/kg of thimerosal was subcutaneously injected into the mouse, the mercury content in the cerebrum was significantly higher 48 h after the thimerosal injection with a maximum peak after 72 h. In addition, mercury content in the cerebrum was still higher on day 7 than in the control group. When lipopolysaccharide was pre-injected into mice to induce damage on blood–brain barrier, the mercury content in the cerebrum was significantly higher at 24 and 72 h after the injection of 12 μg/kg of thimerosal compared to the control group, this dose alone does not cause any increase. The mercury content in the cerebrums of mice was decreased to the control group level on day 7 when a chelator, dimercaprol, was administered once a day from days 3 to 6 after a 60 μg/kg, s.c. injection. In addition, D-penicillamine as a chelator decreased the mercury contents in the cerebrum after the high dose administration. In conclusion, a physiological dose of thimerosal did not increase the content of mercury in the cerebrum, but levels were increased when damage to the blood–brain barrier occurred in mice injected with thimerosal. In addition, a chelator of heavy metals may be useful to remove mercury from the cerebrum.

Keywords: Mercury; Cerebrum; Thimerosal; Lipopolysaccharide; Dimercaprol; D-Penicillamine

1. Introduction

Although thimerosal is the most well established preservative reagent for vaccines, it is a mercuric compound and ethyl and inorganic mercury can be produced from thimerosal in organs (Clarkson, 2002; Burbacher et al., 2005). Organic mercury, including ethyl mercury, can damage cells and tissues (Ueda-Ishibashi et al., 2004; Yel et al., 2005; Slodownik and Ingber, 2005; Havarinasab and Hultman, 2006; Zarini et al., 2006). Furthermore, methyl mercury can cause severe damage in the central nervous system and a relationship between thimerosal use and autism has long been suspected (Stajich et al., 2000; Bernard et al., 2002; Pichichero et al., 2002; François et al., 2005; Mutter et al., 2005). However, the concentration of mercury in the blood of infants and children receiving vaccines with thimerosal has been reported to be very low, without any toxic effects (Madsen et al., 2003; Bigham and Copes, 2005; Clements and McIntyre, 2006), and the agent is still recommended as a cheap and stable preservative for vaccines. Countries such as Japan and United States are now tending to reduce application of thimerosal as much as possible, but it continues to be employed for influenza, tetanus, hepatitis B, poliomyelitis, and measles vaccines in Japan. Clearly, there is a need for more detailed research on the effects of thimerosal in the body, especially in central nervous system.

While the content of mercury in the brain of experimental animals was not found to increase after thimerosal injection (Burbacher et al., 2005), it is not clear whether adverse effects may occur under different circumstances. We previously observed that lipopolysaccharide (LPS) induction of inflammation is associated with an increase in the permeability of the blood–brain barrier (Minami et al., 1996, 1998a,b, 2002). The aim of the present study was to determine whether injection of LPS into mice exerts any effect on mercury content in

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Expression of metallothionein mRNAs on mouse cerebellum microglia cells by thimerosal and its metabolites

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ABSTRACT

Effects of thimerosal and its metabolites, ethyl mercury and thiosalicylate, on the expression of metallothionein (MT) mRNAs in mouse cerebellum microglia cell line, C8-B4 cells, were studied. The level of MT-1 mRNA significantly decreased at early hours and recovered time-dependently 24 h after thimerosal was added to the C8-B4 cells. However, MT-2 and MT-3 mRNA expressions did not change from the control group. In contrast, the expression of MT-1 mRNA increased in a mouse neuroblastoma cell line 6 h after incubation with thimerosal. In addition, the level of MT-1 mRNA decreased in C8-B4 cells 6 h after the addition of thiosalicylate, but ethyl mercury induced MT-1 mRNA expression. When cell viability was compared with thimerosal, thiosalicylate, and ethyl mercury, the viability of C8-B4 cells decreased dose-dependently 24 h after either thimerosal or ethyl mercury was added; however, the viability increased dose-dependently until 15 μM thiosalicylate was added. From the present results, it is concluded that the expression of MT-1 mRNA may be mediated by different factors than the expression of MT-2 mRNA in C8-B4 cells. The reduction of MT-1 mRNA level by thiosalicylate may affect the proliferation of C8-B4 cells.

1. Introduction

Thimerosal is recommended as a preservative of vaccines and toxoids (Geier et al., 2007), because it is cheap with supposedly stable preservative action without side effects. However, as thimerosal splits into two compounds, ethyl mercury and thiosalicylate, in the body (Clarkson, 2002), it was suspected that thimerosal induced autism (Stajich et al., 2000; Bernard et al., 2001; Pichichero et al., 2002; Francois et al., 2005; Mutter et al., 2005). This suspicion was rejected by large-scale epidemiological surveys (Madsen et al., 2003; Clements and McIntyre, 2006; Geier and Geier, 2006; Gallagher and Goodman, 2008; Young et al., 2008), but ethyl mercury has an adverse effect on the central nervous system even at clinical doses of thimerosal (Guzzi and La Porta, 2008).

Metallothionein (MT) is a ubiquitous low-molecular-mass protein. Of the four major isoforms of MT, MT-1 and MT-2 are known to be acute-phase proteins and are induced together in the same tissues in response to various stimuli (Waalkes, 1996; Moffatt and Denizeau, 1997; Miles et al., 2000; Coyle et al., 2002; Haq et al., 2003), and in the brain, it is known that MT-1 and MT-2 are observed in glia and astrocyte cells and MT-3 in neurons (Nishimura et al., 1992; Coyle et al., 2002; Ghazi et al., 2006). For example, MT-1 and MT-2 are induced simultaneously in the brain when either mercury vapor or methyl mercury affects the brain (Leyshon-Sørland et al., 1994; Yasutake et al., 2003, 2004), and both MTs probably act as detoxification substances (Penkowa and Hidalgo, 2000; Penkowa et al., 2006). In contrast, brain MT-3 acted to protect and repair neurons, but recently, it was reported that MT-3 content increased by IL-3, TGF-α, and EFG and decreased by IL-6, kainate, and dexamethasone, and MT-3 content changed in neuronal diseases such as Parkinson and prion diseases (Yu et al., 2001; Carrasco et al., 2003; Hozumi et al., 2006; Kim et al., 2008).

We previously reported that mercury contents increased in the cerebrum when a high dose of thimerosal was subcutaneously injected into mice, and mercury contents increased in the cerebrum after lipopolysaccharide pretreatment even when the dose of thimerosal was low (Minami et al., 2007). In addition, both MT-1 mRNA and protein levels increased in the cerebellum of mice after the injection of low dose of thimerosal (Minami et al., 2009). It seems that thimerosal and its metabolites enter in a brain even if the injection dose is low; therefore, it is important to clarify the effect of thimerosal on the brain.

Microglia and oligodendrocytes were reported to be devoid of MT-1 and MT-2 (Young et al., 1991; Nishimura et al., 1992; Blaauwgeers et al., 1993; Nakajima and Suzuki, 1995), but Vanguri (1995) demonstrated the presence of MT-2 mRNA in a primary culture of microglia, and Agullo et al. (1998) reported that MT-1 and MT-2 in a primary culture of rat microglia were induced by

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Induction of metallothionein in mouse cerebellum and cerebrum with low-dose thimerosal injection

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Abstract Thimerosal, an ethyl mercury compound, is used worldwide as a vaccine preservative. We previously observed that the mercury concentration in mouse brains did not increase with the clinical dose of thimerosal injection, but the concentration increased in the brain after the injection of thimerosal with lipopolysaccharide, even if a low dose of thimerosal was administered. Thimerosal may penetrate the brain, but is undetectable when a clinical dose of thimerosal is injected; therefore, the induction of metallothionein (MT) messenger RNA (mRNA) and protein was observed in the cerebellum and cerebrum of mice after thimerosal injection, as MT is an inducible protein. MT-1 mRNA was expressed at 6 and 9 h in both the cerebrum and cerebellum, but MT-1 mRNA expression in the cerebellum was three times higher than that in the cerebrum after the injection of 12 μg/kg thimerosal. MT-2 mRNA was not expressed until 24 h in both organs. MT-3 mRNA was expressed in the cerebellum from 6 to 15 h after the injection, but not in the cerebrum until 24 h. MT-1 and MT-3 mRNAs were expressed in the cerebellum in a dose-dependent manner. Furthermore, MT-1 protein was detected from 6 to 72 h in the cerebellum after 12 μg/kg of thimerosal was injected and peaked at 10 h. MT-2 was detected in the cerebellum only at 10 h. In the cerebrum, little MT-1 protein was detected at 10 and 24 h, and there were no peaks of MT-2 protein in the cerebrum. In conclusion, MT-1 and MT-3 mRNAs but not MT-2 mRNA are easily expressed in the cerebellum rather than in the cerebrum by the injection of low-dose thimerosal. It is thought that the cerebellum is a sensitive organ against thimerosal. As a result of the present findings, in combination with the brain pathology observed in patients diagnosed with autism, the present study helps to support the possible biological plausibility for how low-dose exposure to mercury from thimerosal-containing vaccines may be associated with autism.

Keywords Thimerosal · Ethyl mercury · Metallothionein · Cerebellum · Cerebrum · Capillary zone electrophoresis

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Introduction

Thimerosal is used as a preservative agent for vaccines and toxoids (Geier et al. 2007). Thimerosal is divided into two components, thiosalicylate and

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Original article

Antineuronal antibodies in autistic children: relation to blood mercury

Background: *It was recently suggested that autism, a severe neurodevelopmental disorder, may involve an autoimmune pathogenesis. Mercury (Hg) is a potential risk factor for autoimmunity in autistic children.*
Objective: *We sought to investigate the expression of antineuronal antibodies, as an index of autoimmunity to brain, in autistic children. The potential relationship between blood mercury and these antibodies was also investigated.*
Methods: *Forty autistic children (20 with mild to moderate and 20 with severe disease) were studied in comparison to 40 healthy children. After complete clinical and neuropsychiatric evaluation, serum antineuronal antibodies and blood Hg levels were estimated.*
Results: *Autistic children had significantly higher seropositivity for antineuronal antibodies (67.5%) than healthy controls (5%). Similarly, the former group had significantly higher blood Hg levels than the latter ($p<0.0001$). Seropositivity of antineuronal antibodies had a significant positive association with elevated blood Hg, which was found in 70% of autistic children, ($p<0.0001$). In addition, the two markers were positively associated with some parameters such as the family history of autoimmunity, autistic severity and some important clinical manifestations of autism (mental retardation, behavioral abnormalities and autistic regression) as well as EEG abnormalities.*
Conclusion: *Autism may be, in part, one of the pediatric autoimmune neuropsychiatric disorders. Such autoimmunity may be triggered by environmental Hg exposure. Further studies are warranted to enforce these concepts. If these assumptions could be proved, routine assessment of serum antineuronal antibodies and blood mercury in autistic children would be mandatory. Studies assessing the role of immunotherapy and Hg chelators as new therapeutic modalities for autism are also recommended.*

Keywords: Antineuronal antibodies; autism; autoimmunity; children; heavy metals; EEG; mercury.

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INTRODUCTION

Autism is a severe neurodevelopmental disorder characterized by impaired communication, social interaction and imagination that is often accompanied by repetitive and stereotyped behavior[1]. It develops before the 36 month of age and persists into adulthood causing life long disability[2]. The prevalence of autism has surged in recent years[3]. The etiology and pathogenesis of autism is not well understood[4]. In view of the possible multifactorial cause, autism can occur as a result of environmental neurotoxicant mercury (Hg) exposure in presence of genetic predisposition[5].

Several sources of toxic Hg exposure in children have been reported in literature: (1) ethyl mercury, which has been the subject of recent scientific inquiry in relation to the controversial pediatric vaccine preservative thimerosal; (2) methyl mercury, is most commonly the result of consumption of contaminated food, particularly fish; (3) inorganic Hg, through the use of topical Hg-based skin creams and in infant teething powders; (4) metalic Hg in dental amalgams, which release Hg vapors[6]. In 2006, Palmer and associates[7] reported that for each 1.000 Ib of environmentally released Hg, there was a 61% increase in the rate of autism. Thus, a logic step is to identify the source of Hg exposure in the child population and consider prevention and control of environmental pollution[5]. Recently, thimerosal was removed from the vaccines in USA, but it is still present in developing countries[8]. However, the issue of the risk of vaccination remains a philosophical one, since to date, the advantage of this policy have not been refuted, while the risk for autoimmune disease has

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ORIGINAL ARTICLE

The levels of blood mercury and inflammatory-related neuropeptides in the serum are correlated in children with autism spectrum disorder

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Abstract Tachykinins (substance P, neurokinin A, and neurokinin B) are pro-inflammatory neuropeptides that may play an important role in some autoimmune neuroinflammatory diseases, including autism spectrum disorder (ASD). Mercury (Hg) is a neurotoxicant, and potentially one of the main environmental triggers for ASD as it induces neuroinflammation with a subsequent release of neuropeptides. This is the first study to explore the potentially causal relationship between levels of serum neurokinin A and blood mercury (BHg) in children with ASD. Levels of serum neurokinin A and BHg were measured in 84 children with ASD, aged between 3 and 10 years, and 84 healthy-matched children. There was a positive linear relationship between the Childhood Autism Rating Scale (CARS) and both serum neurokinin A and BHg. ASD children had significantly higher levels of serum neurokinin A than healthy controls ($P<0.001$). Increased levels of serum neurokinin A and BHg were respectively found in 54.8 % and 42.9 % of the two groups. There was significant and positive linear relationship between levels of serum neurokinin A and BHg in children with moderate and severe ASD, but not in healthy control children. It was found that 78.3 % of the ASD patients with increased serum levels of neurokinin A had elevated BHg levels ($P<0.001$). Neuroinflammation, with increased levels of neurokinin A, is seen in some children with ASD, and may be caused by elevated BHg levels. Further research is recommended to determine the pathogenic role of increased levels of serum neurokinin A and BHg in ASD. The therapeutic role of tachykinin receptor antagonists, a potential new class of anti-inflammatory medications, and Hg chelators, should also be studied in ASD.

Keywords Autism · Neurokinin A · Neuroinflammation · Mercury

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Introduction

Neurogenic inflammation is a neurally mediated immune inflammation that is orchestrated by a large number of neuropeptides, mainly tachykinins. Tachykinins (substance P, neurokinin A, and neurokinin B) have been considered as a group of neuropeptides which are released from the excitatory part of the nonadrenergic, noncholinergic excitatory nervous system nerves after exposure to allergens. The biological activity of tachykinins depends on their interaction with three specific tachykinin receptors, neurokinin (NK)1 (specific for substance P), NK2 (specific for neurokinin A) and NK3 (specific for neurokinin B) receptors (Maggi 2000; Richardson and Vasko 2002; Geppetti et al. 2008; Ramalho et al. 2011; Almeida et al. 2004).

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Neonatal exposure to Thimerosal from vaccines and child development in the first 3 years of life

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ABSTRACT

Background: Despite the common use of Thimerosal as a preservative in childhood vaccines since the 1930s, there are not many studies on ethylmercury toxicokinetics and toxicodynamics in infants. The knowledge of ethylmercury's potential adverse effects is derived mostly from parallel methylmercury research or from animal and theoretical models.

Aim of the study: This study was designed to examine the relationship between neonatal exposure to Thimerosal-containing vaccine (TCV) and child development.

Material and methods: The study sample consisted of 196 infants born between January 2001 and March 2003 to mothers attending ambulatory prenatal clinics in the first and second trimesters of pregnancy in Krakow. Vaccination history (date and the type of the vaccine) was extracted from physicians' records. Child development was assessed using the Bayley Scales of Infant Development (BSID-II) measured in one-year intervals over 3 years. General Linear Model (GLM) and Generalized Estimating Equation (GEE) models adjusted for potential confounders were used to assess the association.

Results: An adverse effect of neonatal TCV exposure was observed for the psychomotor development index (PDI) only in the 12th and 24th months of life ($\beta = -6.44$, $p<0.001$ and $\beta = -5.89$, $p<0.001$). No significant effect of neonatal TCV exposure was found in the 36th month. The overall deficit in the PDI attributable to neonatal TCV exposure measured over the course of the three-year follow-up (GEE) was significantly higher in TCV group ($\beta = -4.42$, $p = 0.001$). MDI scores did not show the adverse association with neonatal TCV exposure.

1. Introduction

The discussion on the safety of Thimerosal-containing vaccines (TCVs) started in 1999 (Joint Statement issued by the American Academy of Pediatrics and the U.S. Public Health Service in July 1999). It was suspected that ethylmercury contained in the vaccines had a harmful effect on children's neurodevelopment. In fact, the toxicity of low dose human exposure to ethylmercury has not been assessed sufficiently, although it is assumed to have a similar effect as exposure to methylmercury (Pichichero et al., 2000, 2002), an organic compound with demonstrated harmful consequences (Grandjean et al., 1997, 2010; Jedrychowski et al., 2006; Lederman et al., 2008; Oken et al., 2008). A joint statement issued by the American Academy of Pediatrics and the U.S. Public Health Service in July 1999 called for measures to remove Thimerosal from vaccines for infants. Since 2004, no vaccines recommended and routinely used in the United States and the European Union (EU) to protect preschool children have contained Thimerosal (CDC, 2004; EMEA, 2001). Nevertheless, most countries continue to use TCVs in their childhood immunization schedules. The World Health Organization's (WHO) position is based on the high effectiveness of vaccines in protecting children against infectious diseases and not on specific studies addressing ethylmercury in population studies (WHO, 2000, 2006). It is difficult to argue with the WHO's strategy, since there is a scarcity of evidence for the harmful influence of ethylmercury in population studies. Reports on the adverse health effects of ethylmercury derive mainly from animal experiments (Hornig et al., 2004; Hewitson et al., 2010) and in vitro tests in human cell-cultures (Mutkus et al., 2005; Yel et al., 2005; Herdman et al., 2006). These experimental studies have shown consistent toxicity in neural cells caused by Thimerosal at concentrations relevant to vaccines. Furthermore, in animal studies, behavior changes in groups exposed to ethylmercury were observed (Hornig et al., 2004; Hewitson et al., 2010). The applicability of these studies to humans is unknown, but the consistency of their results suggests biological consequences in the neurodevelopment of susceptible infants.

The controversies as to the harmful effects of childhood TCVs and the strategies to abandon TCV vaccinations in developed countries create confusion among parents and health workers in some regions where TCVs are still administered to children. In Poland, newborns and infants

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Short Communication

Inhibition of the human erythrocytic glutathione-*S*-transferase T1 (GST T1) by thimerosal

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Abstract

We have investigated the interaction of thimerosal, a widely used antiseptic and preservative, with the human erythrocytic GST T1 (glutathione-*S*-transferase T1). This detoxifying enzyme is expressed in the erythrocytes of solely the human species and it displays a genetic polymorphism. Due to this polymorphism about 25 % of the individuals of the caucasian population lack this activity ("non-conjugators"), while 75 % show it ("conjugators") (Hallier, E., et al., 1993).

Using our newly developed HPLC-fluorescence detection assay (Müller, M., et al., 2001) we have profiled the kinetics of enzyme inhibition in erythrocyte lysates of two individuals previously identified as "normal conjugator" (medium enzyme activity) and "super-conjugator" (very high activity). For the normal conjugator we have determined a 2.77 mM thimerosal concentration to inhibit 50 % of the GST T1 activity. In the case of the super-conjugator a 2.3 mM thimerosal concentration causes a 50 % inhibition of the enzyme activity. For both phenotypes a 14.8 mM thimerosal concentration results in residual enzyme activities equal to those typically detected in non-conjugator lysates. Thus, sufficiently high doses of thimerosal may be able to change the phenotypic status of an individual – at least in vitro – by inhibition of the GST T1 enzyme.

Key words: Enzyme inhibition – glutathione-*S*-transferase T1 – thimerosal – polymorphism – HPLC-fluorescence detection assay

Introduction

Thimerosal is a water-soluble mercury containing derivative of thiosalicylic acid. Due to its antibacterial and antifungal properties it is widely used as an antiseptic agent and as a preservative in topical medication, cleaning solutions for eye lenses, cosmetics, and vaccines. Thimerosal-containing products often lead to sensitization, which may incidentally result in contact dermatitis. In addition, on the cellular basis – at least in vitro –, thimerosal reacts with sulfhydryl groups, as calcium mobilizer and cell function modulating agent. Surprisingly, little is known about its interaction with enzymes. One of the few examples described is the inhibition of some enzymes of the arachidonic acid pathway, such as acyltransferase and lipoxygenase (Stüning, M., et al., 1988).

We have investigated the interaction of thimerosal with the human erythrocytic GST T1 (glutathione-*S*-transferase T1). This generally detoxifying enzyme is expressed in the erythrocytes of solely the human spe-

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In Vitro Uptake of Glutamate in GLAST- and GLT-1-Transfected Mutant CHO-K1 Cells Is Inhibited by the Ethylmercury-Containing Preservative Thimerosal

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ABSTRACT

Thimerosal, also known as thimersal, Merthrolate, or sodiumethylmercurithiosalicylate, is an organic mercurial compound that is used in a variety of commercial as well as biomedical applications. As a preservative, it is used in a number of vaccines and pharmaceutical products. Its active ingredient is ethylmercury. Both inorganic and organic mercurials are known to interfere with glutamate homeostasis. Brain glutamate is removed mainly by astrocytes from the extracellular fluid via high-affinity astroglial Na^+-dependent excitatory amino acid transporters, glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1). The effects of thimerosal on glutamate homeostasis have yet to be determined. As a first step in this process, we examined the effects of thimerosal on the transport of [^{3}H]-D-aspartate, a nonmetabolizable glutamate analog, in Chinese hamster ovary (CHO) cells transfected with two glutamate transporter subtypes, GLAST (EAAT1) and GLT-1 (EAAT2). Additionally, studies were undertaken to determine the effects of thimerosal on mRNA and protein levels of these transporters. The results indicate that thimerosal treatment caused significant but selective changes in both glutamate transporter mRNA and protein expression in CHO cells. Thimerosal-mediated inhibition of glutamate transport in the CHO-K1 cell line DdB7 was more pronounced in the GLT-1-transfected cells compared with the GLAST-transfected cells. These studies suggest that thimerosal accumulation in the central nervous system might contribute to dysregulation of glutamate homeostasis.

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REVIEW ARTICLE

Mercury and autism: Accelerating Evidence?

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Key words: **autism; developmental disorders; ethyl mercury; dental amalgam; mercury; thimerosal; neurotoxicity; estrogen; testosterone; methylation; glutathion**

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Abstract

The causes of autism and neurodevelopmental disorders are unknown. Genetic and environmental risk factors seem to be involved. Because of an observed increase in autism in the last decades, which parallels cumulative mercury exposure, it was proposed that autism may be in part caused by mercury. We review the evidence for this proposal. Several epidemiological studies failed to find a correlation between mercury exposure through thimerosal, a preservative used in vaccines, and the risk of autism. Recently, it was found that autistic children had a higher mercury exposure during pregnancy due to maternal dental amalgam and thimerosal-containing immunoglobulin shots. It was hypothesized that children with autism have a decreased detoxification capacity due to genetic polymorphism. In vitro, mercury and thimerosal in levels found several days after vaccination inhibit methionine synthetase (MS) by 50%. Normal function of MS is crucial in biochemical steps necessary for brain development, attention and production of glutathione, an important antioxidative and detoxifying agent. Repetitive doses of thimerosal leads to neurobehavioral deteriorations in autoimmune susceptible mice, increased oxidative stress and decreased intracellular levels of glutathione in vitro. Subsequently, autistic children have significantly decreased level of reduced glutathione. Promising treatments of autism involve detoxification of mercury, and supplementation of deficient metabolites.

Abbreviations

MTHFR	– methylene tetrahydrofolate reductase
Hg	– mercury
DMSA	– dimercaptosuccinic acid
DMPS	– sodium 2,3-dimercapto-1-propane sulfonate
MS	– methionine synthetase
ASD	– autism spectrum disorders

Introduction

Autism spectrum disorders (ASD), first described in 1943 in eleven children born in the 1930s, have increased worldwide [1,2,3,4]. All forms of mercury are neurotoxic, especially during brain development [5,6]. Thus, some authors

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Comments on the Article "The Toxicology of Mercury and Its Chemical Compounds" by Clarkson and Magos (2006)

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Clarkson and Magos (2006) provide their perspectives on the toxicology of mercury vapor and dental amalgam. As scientists who are involved in preparing a German federal guidline regarding dental amalgam, we welcome additional scientific data on this issue. However, Clarkson and Magos do not present all the relevant studies in their review. The additional data provided here show that: (a) Dental amalgam is the main source of human total mercury body burden, because individuals with amalgam have 2–12 times more mercury in their body tissues compared to individuals without amalgam; (b) there is not necessarily a correlation between mercury levels in blood, urine, or hair and in body tissues, and none of the parameters correlate with severity of symptoms; (c) the half-life of mercury deposits in brain and bone tissues could last from several years to decades, and thus mercury accumulates over time of exposure; (d) mercury, in particular mercury vapor, is known to be the most toxic nonradioactive element, and is toxic even in very low doses, and (e) some studies which conclude that amalgam fillings are safe for human beings have important methodogical flaws. Therefore, they have no value for assessing the safety of amalgam.

Keywords Amalgam, Autism, Ethylmercury, Mercury, Toxicity, Thimerosal

INTRODUCTION

In their, 2006 article, Clarkson and Magos (2006) provide their perspectives on the toxicology of mercury vapor and dental amalgam. In the following comments, we challenge some of the conclusions of Clarkson and Magos on the basis of new scientific literature.

SIGNIFICANCE OF DENTAL AMALGAM FOR MERCURY BODY BURDEN

Dental amalgam is the main source of mercury body burden, as studies in animals (Danscher et al., 1990; Galic et al., 1999, 2001, Hahn et al., 1989, 1990; Lorscheider et al., 1995; Lorscheider and Vimy, 1991; Vimy et al., 1990) and humans show. An approximate 2–5-fold increase of the mercury level in blood und urine as well as a 2- to 12-fold increase of the mercury concentration in several body tissues was observed in amalgam bearers (Barregard et al., 1999; Becker et al., 2002, 2003; Drasch et al., 1992, 1994, 1997; Egglestone and Nylander, 1987; Gottwald et al., 2001; Guzzi et al., 2002, 2006; Levey et al., 2004; Lorscheider et al., 1995; Kingmann et al., 1998; Mortada et al., 2002; Nylander, 1986, 1991; Nylander et al., 1987; Pizzichini et al., 2003, Weiner and Nylander, 1993; Zimmer et al., 2002). Also, mercury from maternal amalgam fillings leads to a significant increase of mercury concentration in the tissues and the hair of fetuses and newborn children. Placental, fetal, and infant mercury body burden correlates with the numbers of amalgam fillings of the mothers (Ask et al., 2002 ; Drasch et al., 1994 ;, Holmes et al., 2003 ; Morgan et al., 2002; Takahashi et al., 2001, 2003; Vather et al., 2000; Yoshida et al., 2002, 2004). Mercury levels in amniotic fluid (Luglie et al., 2003) and breast milk (Drasch et al., 1998; Oskarsson et al., 1996; Vimy et al., 1997) are significantly correlated with the number of maternal amalgam fillings. Mercury from amalgam may be transformed into organic mercury compounds by microorganisms in the gastrointestinal tract (Leistevuo et al., 2001; Heintze et al., 1983; Yannai et al., 1991). Leistevuo et al. (2001) found an increase of methylmercury concentration in amalgam bearers of three times compared to persons without amalgam, although frequency and kind of fish consumption were identical in both groups.

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Kawasaki`s Disease, Acrodynia, and Mercury

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Abstract: A superantigen or autoimmunity has been hypothesized to be the main cause of the Kawasaki`s Disease but the etiology is unknown. Medical literature, epidemiological findings, and some case reports have suggested that mercury may play a pathogenic role. Several patients with Kawasaki`s Disease have presented with elevated urine mercury levels compared to matched controls. Most symptoms and diagnostic criteria which are seen in children with acrodynia, known to be caused by mercury, are similar to those seen in Kawasaki´s Disease. Genetic depletion of glutathione S-transferase , a susceptibility marker for Kawasaki´s Disease, is known to be also a risk factor for acrodynia and may also increase susceptibility to mercury . Coinciding with the largest increase (1985-1990) of thimerosal (49.6% ethyl mercury) in vaccines, routinely given to infants in the U.S. by 6 months of age (from 75µg to 187.5µg), the rates of Kawasaki´s Disease increased ten times, and, later (1985-1997), by 20 times. Since 1990 88 cases of patients developing Kawasaki´s Disease some days after vaccination have been reported to the Centers of Disease Control (CDC) including 19% manifesting symptoms the same day. The presented pathogenetic model may lead to new preventive- and therapeutic strategies for Kawasaki´s disease.

Keywords: Kawasaki's disease, mercury, acrodynia, thimerosal, ethyl mercury, methyl mercury, vaccine, dental amalgam.

INTRODUCTION

Kawaski's Disease (KD), first described in Japan (1967), is an acute febrile multiorgan vasculitis, which predominantly (75 – 80%) affects children younger than 5 years. The disease has an increasing frequency and, in developed countries, has surpassed rheumatic fever as the leading cause of acquired heart disease in children. Early intravenous immunoglobulins in combination with acetyl-salicylic acid have significantly reduced the prevalence of coronary artery abnormalities.

There is no test for diagnosing KD; thus the diagnosis is based on clinical signs and symptoms. Despite of this, of all cases atypical ones amount to 10-45%. Interestingly, another childhood disease, acrodynia (AD) shares most of its diagnostic criteria with KD.

By now, the cause of KD is unknown. Antigens from infections as well as superantigens and genetic polymorphisms have been implicated in the etiological hypotheses. In this review of the literature and analysis of the U.S. Vaccine Adverse Effects Reporting System (VAERS), we hypothesize that prenatal and postnatal exposure to mercury (and synergistic toxins) may be a pathogenic factor in KD.

The VAERS database is an epidemiological database that has been maintained by the Centers for Disease Control (CDC) since 1990 as a surveillance tool to evaluate vaccine safety. An examination of the VAERS database (online public access version: http://vaers.hhs.gov/scripts/data.cfm) with reports entered through January, 31, 2008 was undertaken. The keywords: "kawasaki's disease" and "kawasaki's syndrome." were used. An additional search for "mucocutaneous lymph node syndrome" did not yield any results. The strength of the VAERS database stems from its large reporting base. Its potential weakness is that all vaccine-associated adverse events experienced are not reported.

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ACRODYNIA, KAWASAKI`S DISEASE AND MERCURY

AD was considered a mysterious, systemic disorder, mainly affecting children under the age of five. At its epidemic height (1880-1950), it affected about one in 500 children in industrialized nations [1].

The onset of AD is characterized by high fever lasting more than 5 days; a varying rash such as erythematous plaques, or appearing as measles or scarlet fever; swollen lymph nodes, particularly in the neck; bright red, swollen hands and feet; red, irritated eyes without discharge; bright red, irritated mouth, lips, and throat [2,3]. Neurological, cutaneous, and cardiovascular symptoms are most commonly seen. However, the disease is highly variable; cutaneous symptoms may be mild or lacking while neurological symptoms always seem to be present. It was explained as an infection or nutritional deficiency and it occurred mostly in the teething period [4].

In 1953, as a result of work by Warkany and Hubbard, mercury – coming from teething powders, baby powders, and diapers treated with calomel (85% mercurous chloride) [2] - was accepted as the cause of AD [2]. After a federal ban of these mercury-containing products in 1954, AD disappeared [1]. It should be noted that, *in vitro*, mercurous chloride is one of the least toxic forms of mercury, about 100 times less toxic than are mercury vapor or ethyl mercury contained in vaccines [5]. In addition, it was reported that applications of vaccines (containing ethyl mercury in thimerosal) preceded the onset of AD in several cases [2,3].

KD shares its diagnostic criteria with those of the onset of AD; the two diseases are similar in their clinical appearance. More than 150 symptoms and about 50 laboratory findings which are seen in KD were also described in cases with mercury poisoning (MP), too. (See Table **1**) [2,3,6-57]. KD affects males twice as often as females. This may be explained by *in vitro* studies on human cells which have shown that testosterone synergistically increases the toxicity of mercury, while estrogen protects against mercury toxicity [1].

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Porphyrinuria in childhood autistic disorder: Implications for environmental toxicity

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Abstract

To address a possible environmental contribution to autism, we carried out a retrospective study on urinary porphyrin levels, a biomarker of environmental toxicity, in 269 children with neurodevelopmental and related disorders referred to a Paris clinic (2002–2004), including 106 with autistic disorder. Urinary porphyrin levels determined by high-performance liquid chromatography were compared between diagnostic groups including internal and external control groups. Coproporphyrin levels were elevated in children with autistic disorder relative to control groups. Elevation was maintained on normalization for age or to a control heme pathway metabolite (uroporphyrin) in the same samples. The elevation was significant ($P < 0.001$). Porphyrin levels were unchanged in Asperger's disorder, distinguishing it from autistic disorder. The atypical molecule precoproporphyrin, a specific indicator of heavy metal toxicity, was also elevated in autistic disorder ($P < 0.001$) but not significantly in Asperger's. A subgroup with autistic disorder was treated with oral dimercaptosuccinic acid (DMSA) with a view to heavy metal removal. Following DMSA there was a significant ($P = 0.002$) drop in urinary porphyrin excretion. These data implicate environmental toxicity in childhood autistic disorder.

Keywords: Autism; Asperger; Porphyrin; Mercury; Pervasive Developmental Disorder

Introduction

Autism is a disorder of reciprocal social interaction, behavioral repertoire, and language and communication. Because the phenotype ranges from manifest disability to specific performance elevation, the term Autistic Spectrum Disorder (ASD) (Wing, 1996; Gillberg and Coleman, 2000) is now commonly used to denote the DSM-IV (American Psychiatric Association, 1994) group of pervasive neurodevelopmental disorders encompassing autistic disorder, Asperger's disorder, Rett's disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). A fraction of cases have a defined genetic cause, but the apparent increase in prevalence of ASD (California Department of Human Developmental Services, 2003; Smeeth et al., 2004; Barbaresi et al., 2005), as reviewed (Blaxill, 2004), is suggestive of an environmental contribution. Changes in awareness and diagnostic criteria may explain some of the rise (Croen et al., 2002; Rutter, 2005), but a true increase in prevalence has not been excluded (Rutter, 2005). Elevated ASD rates in urban versus rural areas (Deb and Prasad, 1994; Palmer et al., 2006; Williams et al., 2006) are consistent with an environmental contribution. Several sporadic reports have suggested an association between heavy metal exposure and ASD (Cohen et al., 1982; Accardo et al., 1988; Shannon and Graef, 1996; Lidsky and Schneider, 2005). Superficial similarity between mercury toxicity and ASD has prompted discussion of mercury exposure in the etiology of the disorders (Bernard et al., 2001), while ASD prevalence in Texas schools correlated with local environmental release of mercury (Palmer et al., 2006).

To address an environmental contribution to ASD, several studies have explored the body burden of heavy metals. Because

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Case Report

Thimerosal-Induced Limbal Stem Cell Failure: Report of a Case and Review of the Literature

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Purpose. To report a case of unilateral total limbal stem cell (LSC) failure and corneal opacification secondary to thimerosal- and contact lens–induced ocular surface toxicity. **Methods.** Interventional case report and review of the literature on thimerosal-induced ocular surface changes. **Results.** A 49-year-old woman with a 2-year history of long-term soft contact lens wear developed unilateral total LSC failure and corneal opacification secondary to presumed thimerosal-induced toxicity and contact lens wear. At presentation, best-corrected visual acuities were 20/120 in the right eye and 20/15 in the left eye. The patient underwent a keratolimbal allograft and amniotic membrane graft followed by a penetrating keratoplasty. At the last follow-up, the right eye showed a clear corneal graft with a best-corrected visual acuity of 20/30. **Conclusions.** Thimerosal toxicity can lead to total LSC failure with secondary corneal vascularization and opacification. Keratolimbal allograft followed by penetrating keratoplasty can be successful in reconstructing the ocular surface in such cases.

Key Words: Contact lens—Keratolimbal allograft—Limbal stem cell failure—Preservative—Thimerosal.

Thimerosal is an organic mercurial compound commonly used as an antimicrobial preservative in contact lens disinfecting solutions. Adsorption of thimerosal by a contact lens followed by prolonged contact with the corneal epithelium can potentially cause serious ocular surface damage. Several in vitro studies have shown its cytotoxic effects.[1–4] In the late 1980s, thimerosal was increasingly withdrawn from contact lens solutions because of reports of toxic and immunoallergic ocular surface changes, such as a punctate coarse keratopathy, pseudodendritic lesions, superior limbic keratoconjunctivitis, and a more diffuse keratoconjunctivitis.[5–9] This report describes a case of thimerosal-induced total limbal stem cell (LSC) failure in a young woman. The ocular surface was reconstructed with a keratolimbal allograft (KLAL) and an amniotic membrane graft followed by penetrating keratoplasty. The literature on thimerosal-induced ocular surface changes and management is reviewed.

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CASE REPORT

A 49-year-old myopic woman with a 2-year history of soft contact lens wear was referred with a history of recurrent episodes of ocular discomfort in her right eye with markedly decreased vision. Since beginning contact lens wear, she was repeatedly examined by her optometrist for redness, discomfort, and burning while using her lenses. The contact lenses were changed without benefit until they were eventually discontinued 1 year before her presentation, with some improvement of symptoms in her right eye and resolution of symptoms in her left eye. The patient had been using an Alcon saline solution (Fort Worth, TX) to clean and store her soft contact lenses. This product is a sterile, buffered, isotonic aqueous solution preserved with thimerosal, sorbic acid, and edetate disodium. Even after detailed questioning, the patient could not recollect the name of the soft contact lenses she had been using. The patient had no other preexisting ocular or medical conditions that could have predisposed to LSC failure.

The vision in her right eye gradually deteriorated with worsening of symptoms until she was referred for recurrent episodes of redness, blurred vision, and pain in the right eye. Her best-corrected visual acuity was 20/120 in the right eye and 20/15 in the left eye. The right cornea showed extensive epithelial and subepithelial haze with superficial and anterior stromal vascularization. There was patchy uptake of fluorescein across the whole cornea and loss of all recognizable limbal palisades of Vogt, clinically consistent with total LSC failure (Fig. 1). There was no evidence of tear film dysfunction. The left cornea appeared healthy. The condition in the right eye was thought to be secondary to recurrent or persistent keratoconjunctivitis related to thimerosal preservative and contact lens wear.

After receiving informed consent, a KLAL with an amniotic membrane patch graft was performed to repopulate the LSCs (Fig. 2). A conjunctival limbal autograft was not performed because of suspicion of subclinical LSC damage in the left eye. Postoperatively, the patient was immunosuppressed with oral cyclosporine for a period of 6 months. The postoperative recovery was uneventful. Penetrating keratoplasty was performed 17 months later.

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Quantitative Bioimaging to Investigate the Uptake of Mercury Species in *Drosophila melanogaster*

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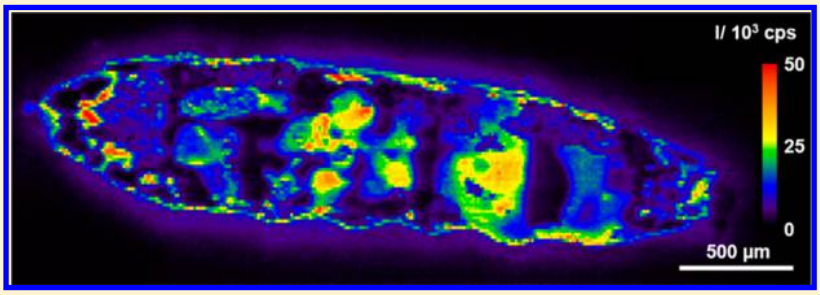

ABSTRACT: The uptake of mercury species in the model organism *Drosophila melanogaster* was investigated by elemental bioimaging using laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS). The mercury distribution in *Drosophila melanogaster* was analyzed for the three species mercury(II) chloride, methylmercury chloride, and thimerosal after intoxication. A respective analytical method was developed and applied to the analysis of the entire *Drosophila melanogaster* first, before a particular focus was directed to the cerebral areas of larvae and adult flies. For quantification of mercury, matrix-matched standards based on gelatin were prepared. Challenges of spatially dissolved mercury determination, namely, strong evaporation issues of the analytes and an inhomogeneous distribution of mercury in the standards due to interactions with cysteine containing proteins of the gelatin were successfully addressed by complexation with *meso*-2,3-dimercaptosuccinic acid (DMSA). No mercury was detected in the cerebral region for mercury(II) chloride, whereas both organic species showed the ability to cross the blood−brain barrier. Quantitatively, the mercury level in the brain exceeded the fed concentration indicating mercury enrichment, which was approximately 3 times higher for methylmercury chloride than for thimerosal.

The heavy metal mercury has been known as a toxic element for centuries.[1,2] The public interest for mercury intoxication and mercury related diseases has increased during the last decades starting with the *Minamata disaster* in the 1950s and the *Iraq poison grain disaster* in 1971.[3−7] The reflection of these demonstrated that the toxicity of mercury is significant depending on its chemical species.

Generally, there are three main categories of mercury species including elemental (Hg^0), inorganic (Hg^{2+}, Hg_2^{2+}) and organic (e.g., $MeHg^+$, $EtHg^+$, $PhHg^+$) mercury.[1,8] Regarding the physical and chemical properties and especially the toxicology of the different species, it is obvious that medical or toxicological assumptions can only be generated via speciation analysis.[9,10] The global existence of mercury in the environment is based on both natural (e.g., thermal evaporation from oceans and land masses, biomass burning, and emissions of volcanoes) and anthropogenic sources (e.g., fossil fuel combustion, chlor-alkali electrolysis plants, artisanal gold mining, cement production, waste incineration, and dental amalgam) of which the anthropogenic emissions represent the greater part and mainly originate from Asia.[6,11−13] Because of the long lifetime and persistence of mercury in the atmosphere and hydrosphere, mercury can be transported over large distances leading to a global distribution. Thus, biotransformation of mercury in aquatic ecosystems into organic mercury species via methylation is a process that leads to the formation of highly toxic methylmercury.[14−16] In the case of organic mercury species, a large accumulation in the food chain can be observed. Fish consumption, especially of long-lived and predatory fish like tuna and shark, is one of the main sources of human mercury exposure and even culinary treatment does not show a significant decrease of mercury species.[14,15,17−19]

Furthermore, rice as a staple food may represent another important source of human mercury uptake due to various and diffuse mercury pollution. Hence, Meng et al. recently described the speciation and localization of mercury in rice grain (sampled from a Hg-contaminated region in China).[20]

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EFFECTS OF THIMEROSAL, AN ORGANIC SULFHYDRYL MODIFYING AGENT, ON SEROTONIN TRANSPORT ACTIVITY INTO RABBIT BLOOD PLATELETS

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Abstract—The effects of the sulfhydryl group inhibitor thimerosal on serotonin (5-HT) transport activity into rabbit blood platelets were investigated, along with its effects on the intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$). ^{3}H-5-HT transport activity into rabbit blood platelets was inhibited by treatment with 10^{-5} M thimerosal for 30 min, which did not cause 5-HT release from platelets. The thimerosal-induced inhibition of 5-HT transport was antagonized by dithiothreitol. It was suggested that the thimerosal acts as a sulfhydryl inhibitor and inhibits 5-HT transport activity independently of the 5-HT release reaction in our experiment using rabbit blood platelets. As aspirin did not affect thimerosal-induced 5-HT transport inhibition, it was suggested that the thromboxane A_2-generating system does not operate in the effect of thimerosal on 5-HT transport into blood platelets. Furthermore, thimerosal induced a transient elevation of $[Ca^{2+}]i$, which was followed by a sustained increase. In the absence of extracellular Ca^{2+}, thimerosal caused only a transient increase in $[Ca^{2+}]i$. It was suggested that the elevation of $[Ca^{2+}]i$ consisted of two phases, e.g. a transient phase induced by Ca^{2+} mobilization from the intracellular store sites and a sustained phase which might be explained by Ca^{2+} influx from the extracellular environment. In conclusion, thimerosal inhibited 5-HT transport into blood platelets at a concentration which did not induce 5-HT release, and intracellular Ca^{2+} mobilization might mediate the inhibitory effect of thimerosal on 5-HT transport. Copyright © 1996 Elsevier Science Ltd.

The organic mercury compound thimerosal is known to modify physiological responses by binding to sulfhydryl and disulfide groups in various cellular systems. In rat hepatocytes, for example, thimerosal promoted the release of Ca^{2+} from inositol trisphosphate-sensitive Ca^{2+} stores (Missiaen *et al.*, 1991). Thimerosal was shown to inhibit Ca^{2+} uptake in skeletal muscle sarcoplasmic reticulum and rat cerebellar microsomes by inhibiting Ca^{2+}-ATPase (Sayers *et al.*, 1993). In addition, it was reported that thimerosal may be an alternative agent for studying Ca^{2+}-induced-Ca^{2+}-release (CICR) in caffeine-insensitive cells such as unfertilized hamster eggs (Swann, 1991) and blood platelets (Adunyah, 1986). Hecker *et al.* (1989) reported that thimerosal caused mobilization of Ca^{2+} from its intracellular stores, and then induced aggregation and 5-HT release in human blood platelet preparations. Thus, thimerosal has been proposed to be a useful agent to analyze intracellular Ca^{2+} movement and its physiological significance in various cellular systems including blood platelets.

5-hydroxytryptamine (5-HT) functions as a neurotransmitter in the mammalian nervous system. 5-HT is known to play an important role in a multitude of cognitive and behavioral (dys)functions including motor control, feeding, anxiety, depression and sexual activity. Regulation of 5-HT transport has been the major focus of antidepressant research, with many specific 5-HT uptake inhibitors being effective antidepressants. Since platelets have a very rapid active transport system for 5-HT which has been shown to have the same pharmacological characteristics as serotonergic nerve endings (Pletscher, 1968), platelets are proposed to be a potential model for 5-HT neurons (Pletscher, 1988). It has been reported that the human platelet 5-HT uptake site is identical to the human brain 5-HT transporter, and that both proteins are encoded by the same single-copy gene which has been assigned to the human chromosome 17 (Lesch *et al.*, 1993). In view of data suggesting abnormal platelet

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Altered Heavy Metals and Transketolase Found in Autistic Spectrum Disorder

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Abstract Autism and autism spectrum disorder (ASD) are developmental brain disorders with complex, obscure, and multifactorial etiology. Our recent clinical survey of patient records from ASD children under the age of 6 years and their age-matched controls revealed evidence of abnormal markers of thiol metabolism, as well as a significant alteration in deposition of several heavy metal species, particularly arsenic, mercury, copper, and iron in hair samples between the groups. Altered thiol metabolism from heavy metal toxicity may be responsible for the biochemical alterations in transketolase, and are mechanisms for oxidative stress production, dysautonomia, and abnormal thiamine homeostasis. It is unknown why the particular metals accumulate, but we suspect that children with ASD may have particular trouble excreting thiol-toxic heavy metal species, many of which exist as divalent cations. Accumulation or altered mercury clearance, as well as concomitant oxidative stress, arising from redox-active metal and arsenic toxicity, offers an intriguing component or possible mechanism for oxidative stress-mediated neurodegeneration in ASD patients. Taken together, these factors may be more important to the etiology of this symptomatically diverse disease spectrum and may offer insights into new treatment approaches and avenues of exploration for this devastating and growing disease.

Keywords Transketolase · Hair · Heavy metal · Copper · Iron · Mercury · Arsenic · Divalent cation · Transport · Autistic spectrum disorder · Mitochondria · Oxidative stress

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Wide Use of Merthiolate May Cause Mercury Poisoning in Mexico

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In 1999, Merthiolate (Thimerosal, Thiomersal), an ethylmercurythiosalicylate preservative, was suddenly spotlighted on the grounds that vaccines containing the preservative might increase the risk of autism and/or other neurodevelopmental disorders (Ball et al. 1999). On the basis of current evidence, however, some of investigators consider it improbable that Merthiolate and autism are linked (Nelson and Bauman 2003). On the other hand, Merthiolate had also been widely used as an antiseptic agent, and is still in daily use mainly as a skin antiseptic especially in developing countries, although there is a downward trend. Indeed, in Mexico Merthiolate had been widely employed as disinfectant for the skin or wound surface in hospitals or clinics in the early 1990s. Therefore, we attempted to examine mercury levels in head hair from Mexican staff engaged in medical service in 1991 and 1992, because ethylmercury has some similarities to methylmercury (Clarkson et al. 2003), of which toxicity is well known as Minamata disease (Harada 1995). In addition, they have continued to use Merthiolate as disinfectant until the present, although the use is also on the decrease. Thus, we report here mercury levels in head hair collected from Mexican medical staff in the early 1990s, albeit rather antiquated.

MATERIALS AND METHODS

In 1991, 12 head hair samples were collected from nurses working in a general hospital (Toluca, Mexico) using Merthiolate. Each hair sample was cut as close to the scalp for both total mercury (T-Hg) and methylmercury (MeHg) analyses. Although MeHg has no direct bearing upon Merthiolate, namely, ethylmercury, we determined the MeHg level for reference. As a result, as mentioned later, seven out of 12 samples showed a high T-Hg level, but the ratio of MeHg to T-Hg was very low. Accordingly, the next year we further measured T-Hg levels, but not MeHg level because of the above low level, in head hair from 27 medical doctors, 34 nurses, and two others (an office worker and an expert of clinical examination) working in three general hospitals also using Merthiolate in Mexico City. In addition, we inquired their subjective symptoms. Informed consent was obtained from all subjects, based upon the Declaration of Helsinki, 1964.

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BRAIN RESEARCH

Research Report

Neonatal administration of a vaccine preservative, thimerosal, produces lasting impairment of nociception and apparent activation of opioid system in rats

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ABSTRACT

Thimerosal (THIM), an organomercury preservative added to many child vaccines is a suspected factor in pathogenesis of neurodevelopmental disorders. We examined the pharmacokinetics of Hg in the brain, liver and kidneys after i.m. THIM injection in suckling rats and we tested THIM effect on nociception. THIM solutions were injected to Wistar and Lewis rats in a vaccination-like mode on PN days 7, 9, 11 and 15 in four equal doses. For Wistar rats these were: 12, 48, 240, 720, 1440, 2160, 3000 μg Hg/kg and for Lewis: 54, 216, 540 and 1080 μg Hg/kg. Pharmacokinetic analysis revealed that Hg from THIM injections accumulates in the rat brain in significant amounts and remains there longer than 30 days after the injection. At the 6th week of age animals were examined for pain sensitivity using the hot plate test. THIM treated rats of both strains and sexes manifested statistically significantly elevated pain threshold (latency for paw licking, jumping) on a hot plate (56 °C). Wistar rats were more sensitive to this effect than Lewis rats. Protracted THIM-induced hypoalgesia was reversed by naloxone (5 mg/kg, i.p.) injected before the hot plate test, indicative of involvement of endogenous opioids. This was confirmed by augmented catalepsy after morphine (2.5 mg/kg, s.c.) injection. Acute THIM injection to 6-week-old rats also produced hypoalgesia, but this effect was transient and was gone within 14 days. Present findings show that THIM administration to suckling or adult rats impairs sensitivity to pain, apparently due to activation the endogenous opioid system.

1. Introduction

Thimerosal (THIM; also known as thiomerosal or sodium ethylmercurithiosalicylate), which contains about 49% of mercury (Hg) by weight, has been used as a vaccine preservative for decades without rigorous studies examining its safety in developing mammalian organism, including infants. A vast body of scientific literature provides evidence that all forms of Hg are highly toxic to animals (rev. Díez, 2009; Clarkson and Magos, 2006). THIM is biotransformed in the body to ethylmercury and subsequently also into inorganic forms of Hg (Qvarnström et al., 2003). Significant amounts of

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Abbreviations: SIB, self injurious behaviors; THIM, thimerosal; Hg, mercury

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ORIGINAL PAPER

Neonatal Administration of Thimerosal Causes Persistent Changes in Mu Opioid Receptors in the Rat Brain

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Abstract Thimerosal added to some pediatric vaccines is suspected in pathogenesis of several neurodevelopmental disorders. Our previous study showed that thimerosal administered to suckling rats causes persistent, endogenous opioid-mediated hypoalgesia. Here we examined, using immunohistochemical staining technique, the density of μ-opioid receptors (MORs) in the brains of rats, which in the second postnatal week received four i.m. injections of thimerosal at doses 12, 240, 1,440 or 3,000 μg Hg/kg. The periaqueductal gray, caudate putamen and hippocampus were examined. Thimerosal administration caused dose-dependent statistically significant increase in MOR densities in the periaqueductal gray and caudate putamen, but decrease in the dentate gyrus, where it was accompanied by the presence of degenerating neurons and loss of synaptic vesicle marker (synaptophysin). These data document that exposure to thimerosal during early postnatal life produces lasting alterations in the densities of brain opioid receptors along with other neuropathological changes, which may disturb brain development.

Keywords Thimerosal · Mu opioid receptors · Rat · Brain · Development

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Introduction

Thimerosal (THIM), an organomercury compound, which contains approximately 49% mercury (Hg) by weight, has been used for decades as a preservative in pediatric vaccines without adequate testing for its safety in developing organisms. THIM is metabolized in the body first into ethylmercury and subsequently into other organic and inorganic mercury forms [1]. Centuries of human experience and a large body of scientific data document that all forms of Hg are highly toxic. Considerable amounts of Hg have been found in the blood of human infants after the injection of THIM-containing vaccines [2, 3] and studies conducted with infant monkeys showed that Hg from THIM-vaccine injections accumulates in the brain at concentrations many times higher than those in the blood, and that it stays there for months or years [4]. Post vaccination levels of Hg in infant brains may reach medium nanomolar concentrations, which are neurotoxic and kill neurons in vitro [5]. THIM doses equivalent to those used in vaccines have been shown to harm the brains of developing mice [6].

Early life exposure to mercurials, including THIM, is suspected to be a pathogenic factor in several neurodevelopmental disorders [7–10]. We have previously shown that THIM administration to suckling rats in a mode similar to infant immunization and at doses analogous to those used in pediatric vaccines, or higher, persistently augments

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Research report

Persistent behavioral impairments and alterations of brain dopamine system after early postnatal administration of thimerosal in rats

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ABSTRACT

The neurotoxic organomercurial thimerosal (THIM), used for decades as vaccine preservative, is a suspected factor in the pathogenesis of some neurodevelopmental disorders. Previously we showed that neonatal administration of THIM at doses equivalent to those used in infant vaccines or higher, causes lasting alterations in the brain opioid system in rats. Here we investigated neonatal treatment with THIM (at doses 12, 240, 1440 and 3000 μg Hg/kg) on behaviors, which are characteristically altered in autism, such as locomotor activity, anxiety, social interactions, spatial learning, and on the brain dopaminergic system in Wistar rats of both sexes. Adult male and female rats, which were exposed to the entire range of THIM doses during the early postnatal life, manifested impairments of locomotor activity and increased anxiety/neophobia in the open field test. In animals of both sexes treated with the highest THIM dose, the frequency of prosocial interactions was reduced, while the frequency of asocial/antisocial interactions was increased in males, but decreased in females. Neonatal THIM treatment did not significantly affect spatial learning and memory. THIM-exposed rats also manifested reduced haloperidol-induced catalepsy, accompanied by a marked decline in the density of striatal D_2 receptors, measured by immunohistochemical staining, suggesting alterations to the brain dopaminergic system. Males were more sensitive than females to some neurodisruptive/neurotoxic actions of THIM. These data document that early postnatal THIM administration causes lasting neurobehavioral impairments and neurochemical alterations in the brain, dependent on dose and sex. If similar changes occur in THIM/mercurial-exposed children, they could contribute do neurodevelopmental disorders.

1. Introduction

Thimerosal (THIM; sodium ethyl-mercurithiosalicylate; containing approximately 49% of mercury (Hg) by weigh), has been added to pediatric vaccines as a preservative since the 1930s (and still is in many developing countries), without being adequately tested for safety in developing organisms. In the body THIM is metabolized first to ethyl-mercury and further to inorganic mercury compounds, which accumulate in the brain and other vital organs [1,2]. With increasing numbers of vaccines injected to progressively younger infants (some only a few hours old), a legitimate concern emerged that Hg from vaccines accumulating in infant brains might contribute to the epidemics of neurodevelopment disorders in children [3–9]. This issue is a subject of hot debates, but still remains controversial.

Concerns related to use of THIM in pediatric vaccines stem primarily from its neurotoxicity, analogous to that of other mercurials. THIM has been shown to kill neurons by apoptosis and necrosis in vitro at nanomolar and low micromolar concentrations, which might be reached in the brain after vaccination [10–15]. The molecular mechanisms of THIM-induced neurotoxicity involve DNA breakage [10,16], depolarization and damage of mitochondrial membranes [11,15], generation of reactive oxygen species, release of cytochrome and apoptosis inducing factors from mitochondria to cytosol, and activation of caspases 9 and 3, known to participate

Abbreviations: THIM, thimerosal; DA, dopamine; ASD, autism spectrum disorders.

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Comparison of organic and inorganic mercury distribution in suckling rat

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ABSTRACT: Thiomersal is used as a preservative in vaccines given to small children. The metabolic product of thiomersal is ethylmercury and its distribution and kinetics are still not known, especially at this early age. The purpose of this study was to compare the body distribution of two forms of mercury: organic (thiomersal) and inorganic (mercury(2+) chloride) in very young, suckling rats. Mercury was applied subcutaneously three times during the suckling period on days 7, 9 and 11 of pups age, imitating the vaccination of infants. A single dose of mercury was equimolar in both exposed groups, i.e. 0.81 μmol Hg kg^{-1}. At 14 days of age the animals were killed and the total mercury analysed in blood and organs (kidney, liver and brain). The analytical method applied was total decomposition, amalgamation, atomic absorption spectrometry. The results showed that the level of mercury was higher in the liver and kidney of the inorganic mercury group than in the thiomersal exposed group. However, the brain and blood concentrations of mercury were higher in the thiomersal exposed group. These results need to be clarified by additional data on the kinetic pathways of ethylmercury compared with inorganic mercury. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: thiomersal; ethylmercury; mercury(2+) chloride; suckling rat; distribution; blood; kidney; liver; brain

Introduction

Thiomersal (Thiomerosal, Merthiolat) is an organic compound of mercury added as a preservative to the childhood vaccines diphtheria-tetanus-pertussis (DTP) or diphtheria-tetanus (DT). The vaccine is given subcutaneously to infants several times during their first 6 months of life, up to the age of 6 years. The metabolite of thiomersal is ethylmercury and its absorption, distribution and excretion is similar to methylmercury. The similarity of metabolic pathways and actions are due to similar reactions as organic molecules. Both substances increase oxidative stress and are neurotoxic (Ueha-Ishibashi *et al*., 2004; ATSDR, 1999). Ethylmercury is, however, less toxic than methylmercury due to the lower clearance half-time and the lower possibility to pass the blood–brain barrier (Magos, 2003) than methylmercury. Due to unknown toxicity from low-dose exposures to ethylmercury, there has been concern that this exposure to mercury may be of some detriment to young children. Autistic spectrum disorders and neurodevelopmental disorders have been a controversial topic since 1999 connected with thiomersal-containing vaccines (Parker *et al*., 2004). Therefore, the American Academy of Pediatrics and the US Public Health Service issued a joint statement calling for the removal of thiomersal from vaccines. However, in our country and probably in many other countries such vaccines are still in use.

Data on the difference between ethylmercury and inorganic mercury distribution in newborns are, however, limited. In this study the distribution of the two chemical forms of mercury, organic (thiomersal) and inorganic (mercury(2+) chloride) was measured and compared in very young, suckling rats. Application of substances was subcutaneous three times during the suckling period, imitating the vaccination of infants.

Methods

Animals and Experimental Protocol

Adult animals used in the experiment were Wistar rats raised in the Institute's breeding farm (Institute for Medical Research and Occupational Health, Zagreb, Croatia). They were fed standard rat diet (Mucedola, Milano, Italy) and tap water *ad libitum*. The rats were kept in the animal facility at a constant room temperature of 20–22 °C, constant humidity (40 ± 10%) and 12 h light/dark cycle. Females were mated with males in the ratio of 3 : 1. Pregnant females were kept in individual

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Environmental mercury release, special education rates, and autism disorder: an ecological study of Texas

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Abstract

The association between environmentally released mercury, special education and autism rates in Texas was investigated using data from the Texas Education Department and the United States Environmental Protection Agency. A Poisson regression analysis adjusted for school district population size, economic and demographic factors was used. There was a significant increase in the rates of special education students and autism rates associated with increases in environmentally released mercury. On average, for each 1000 lb of environmentally released mercury, there was a 43% increase in the rate of special education services and a 61% increase in the rate of autism. The association between environmentally released mercury and special education rates were fully mediated by increased autism rates. This ecological study suggests the need for further research regarding the association between environmentally released mercury and developmental disorders such as autism. These results have implications for policy planning and cost analysis.

Keywords: Mercury; Special education; Autism; Environmental toxins; Ecological

Introduction

Exposure to a variety of environmental neurotoxicants is known to affect normal child development, resulting in a spectrum of adverse outcomes, ranging from severe mental retardation and developmental disability to more subtle changes in functioning, depending in part on the timing and dose of the chemical agent (Landrigan and Garg, 2002; Mendola et al., 2002; Rice and Barone, 2000).

The Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) section 104 (i), as amended by the Superfund Amendments and Reauthorization Act (SARA), requires the Agency for Toxic Substances and Disease Registry (ATSDR) and the Environmental Protection Agency (EPA) to prepare a list, in order of priority, of substances that are most commonly found at waste facilities on the National Priorities List (NPL) and which are determined to pose the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure. Accordingly, mercury is listed as the third-most frequently found (arsenic and lead are

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Proximity to point sources of environmental mercury release as a predictor of autism prevalence

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Abstract

The objective of this study was to determine if proximity to sources of mercury pollution in 1998 were related to autism prevalence in 2002. Autism count data from the Texas Educational Agency and environmental mercury release data from the Environmental Protection Agency were used. We found that for every 1000 pounds of industrial release, there was a corresponding 2.6% increase in autism rates ($p<.05$) and a 3.7% increase associated with power plant emissions($P<.05$). Distances to these sources were independent predictors after adjustment for relevant covariates. For every 10 miles from industrial or power plant sources, there was an associated decreased autism Incident Risk of 2.0% and 1.4%, respectively ($p<.05$). While design limitations preclude interpretation of individual risk, further investigations of environmental risks to child development issues are warranted.

Keywords: Mercury; Autism; Environment; Distance; Industry

Introduction

Mercury is a heavy metal found naturally in trace amounts in the earth's atmosphere in differing forms—as elemental vapor, reactive gaseous compounds, or particulate matter. Studies show that background levels of environmental mercury deposition have steadily increased several fold since the pre-industrial era (Schuster et al., 2002), with the largest source of potentially adverse exposures coming primarily from coal-fired utility plants (33%), municipal/medical waste incinerators (29%) and commercial/industrial boilers (18%)—estimated to be responsible for 158 tons of environmental mercury released per year in the US (Environmental Protection Agency, Report to Congress, 1997). Other sources include hazardous waste sites, cement factories, and chlorine production plants. According to the Agency for Toxic Substances and Disease Registry (ATSDR), next to arsenic and lead, mercury is the third most frequently found toxic substance in waste facilities in the United States (ATSDR, 2001).

Mercury is now widespread in the environment (EPA, 1997; ATSDR, 2001). The long-range atmospheric transport of mercury (Ebinghaus et al., 2001), and its conversion to organic forms through bio-accumulation in the aquatic food chain has been known for some time (MacGregor, 1975; Mahaffey, 1999). Notwithstanding, there are emerging concerns over the potential adverse effects of ambient levels of environmental mercury during early childhood development. There is sufficient evidence that children and other developing organisms are particularly susceptible to the adverse neurological effects of mercury (Landrigan and Garg, 2002; Grandjean et al., 1995; Ramirez et al., 2003; Rice and Barone, 2000).

Evidence from animal studies suggests that neonates lack the ability to efficiently excrete both methylmercury (Rowland et al., 1983) and inorganic mercury (Thomas and Smith, 1979), and that there is a higher lactational transfer of inorganic mercury than methylmercury (Sundberg et al., 1991a, b). Correspondingly, it has been shown that infants exposed via milk from mothers who were accidentally

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Evaluation of Cytotoxicity Attributed to Thimerosal on Murine and Human Kidney Cells

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Renal inner medullary collecting duct cells (mIMCD3) and human embryonic kidney cells (HEK293) were used for cytoscreening of thimerosal and mercury chloride ($HgCl_2$). Thimerosal and $HgCl_2$ acted in a concentration-dependent manner. In mIMCD3 cells the 24-h LC_{50} values for thimerosal, thiosalicylic acid, 2,2-dithiosalicylic acid, and 2-sulfobenzoic acid were 2.9, 2200, >1000, and >10,000 μ*M*, respectively. The 24-h LC_{50} value for $HgCl_2$ in mIMCD3 cells was 40 μ*M*. In HEK293 cells, the 24-h LC_{50} value for thimerosal was 9.5 μ*M*. These data demonstrate that the higher cytotoxicity produced by thimerosal on renal cells with respect to similar compounds without Hg may be related to this metal content. The present study also establishes mIMCD3 cells as a valuable model for evaluation of cytotoxicity of nephrotoxic compounds.

Public health regulatory agencies are increasingly concerned about the impact of renal diseases produced by commercially used compounds that are nephrotoxic (Thadhani et al., 1996). In particular, renal inner medullary cells are often exposed to high concentrations of common nephrotoxic substances and also frequently are subjected to hyperosmotic and ischemic stress (Burg, 2002; Lee et al., 2002). Little is known concerning the cytotoxic effects produced by drugs and toxicants other than nonsteroidal anti-inflammatory drugs (Rocha et al., 2001) on renal inner medullary collecting duct cells (mIMCD3), which are an immortalized cell line derived from mouse renal inner medulla.

Thimerosal (ethylmercurithiosalicylic acid) is a mercury-containing preservative that has been used as an additive for vaccine and biological products for more than 70 years. Thimerosal dissociates as 49.5% ethylmercury by weight and thiosalicylic acid. High-dose, acute or chronic mercury (Hg) exposure of children and adults resulted in nephrotoxicity (Clarkson, 1993; Van Vleet & Schnellmann, 2003). Ethylmercury and thimerosal induce significantly higher Hg concentration in the kidney than in brain (Harry et al., 2004). However, thimerosal has been implicated in neuronal toxicity and autism (Geier & Geier, 2006; Kern & Jones, 2006; Kern et al., 2007).

The colorimetric cell survival assay using the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) has been widely used for measuring cell proliferation and survival (Mosmann, 1983). This assay measures the reduction of a tetrazolium component into an insoluble purple formazan product by the mitochondria of viable cell. The present study was undertaken to investigate cytotoxicity of thimerosal and its structural analogs, and inorganic Hg in mIMCD3 and human embryonic kidney (HEK293) cells.

MATERIALS AND METHODS

Cultures of mIMCD3 and HEK 293 Cells

mIMCD3 cells of passage 19 and HEK293 cells of passage 35 were used for all experiments. All reagents for cell

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Effects of Thimerosal on NGF Signal Transduction and Cell Death in Neuroblastoma Cells

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Signaling through neurotrophic receptors is necessary for differentiation and survival of the developing nervous system. The present study examined the effects of the organic mercury compound thimerosal on nerve growth factor signal transduction and cell death in a human neuroblastoma cell line (SH-SY5Y cells). Following exposure to 100 ng/ml NGF and increasing concentrations of thimerosal (1 nM–10 μM), we measured the activation of TrkA, MAPK, and PKC-δ. In controls, the activation of TrkA MAPK and PKC-δ peaked after 5 min of exposure to NGF and then decreased but was still detectable at 60 min. Concurrent exposure to increasing concentrations of thimerosal and NGF for 5 min resulted in a concentration-dependent decrease in TrkA and MAPK phosphorylation, which was evident at 50 nM for TrkA and 100 nM for MAPK. Cell viability was assessed by the LDH assay. Following 24-h exposure to increasing concentrations of thimerosal, the EC_{50} for cell death in the presence or absence of NGF was 596 nM and 38.7 nM, respectively. Following 48-h exposure to increasing concentrations of thimerosal, the EC_{50} for cell death in the presence and absence of NGF was 105 nM and 4.35 nM, respectively. This suggests that NGF provides protection against thimerosal cytotoxicity. To determine if apoptotic versus necrotic cell death was occurring, oligonucleosomal fragmented DNA was quantified by ELISA. Control levels of fragmented DNA were similar in both the presence and absence of NGF. With and without NGF, thimerosal caused elevated levels of fragmented DNA appearing at 0.01 μM (apoptosis) to decrease at concentrations >1 μM (necrosis). These data demonstrate that thimerosal could alter NGF-induced signaling in neurotrophin-treated cells at concentrations lower than those responsible for cell death.

Key Words: **signal transduction; neurotrophin; mercury compound.**

Thimerosal is an organic mercury compound that is used as a preservative in many vaccines due to its antibacterial and antifungal abilities. It consists of an organic radical, ethylmercury (49.6% by weight), which is bound to the sulfur atom of the thiol group of salicylic acid. The type of anion attached to ethylmercury affects neither the distribution of mercury in the body nor its toxicity (Suzuki and Toyama, 1973; Ulfvarson, 1962), while the organic radical has a strong impact on both (Magos, 2003). Ethylmercury and its decomposition product, Hg^{2+}, rapidly accumulate in the tissues (Magos, 2001), preferentially in the kidneys and brain (Blair *et al.*, 1975). Following *in vivo* administration, ethylmercury passes through cellular membranes and concentrates in cells of vital organs, including the brain, where it releases inorganic mercury, raising its concentrations higher than equimolar doses of its close and highly toxic relative methylmercury (Magos *et al.*, 1985).

There has recently been concern about the effects of this source of mercury on the fetal and infant nervous system, especially in infants who develop neurodevelopmental disorders such as autism, attention deficit-hyperactive disorder (ADHD), and speech or language delay (Bernard *et al.*, 2001; Kidd, 2002). Human studies do not show a significant association between the use of thimerosal-containing vaccines and the development of autism in children. More studies on this topic need to be completed to obtain conclusive results (NAS, 2001).

Some recent *in vitro* studies show that certain concentrations of thimerosal have decreased cellular viability in human neurons and fibroblasts. For example, Baskin *et al.* (2003) noted an increase in membrane permeability to DAPI dye as early as 2 h after incubation of human cortical neurons and fibroblasts with 250 μM thimerosal. A 6 h incubation resulted in membrane damage (loss of DAPI dye exclusion), DNA breaks, and apoptosis as indicated by morphology and caspase-3 activation (Baskin *et al.*, 2003; Makani *et al.*, 2002).

The studies cited above identified a number of molecular targets for thimerosal, including micronuclei induction, disturbances of intracellular calcium, and inhibition of glutathione content (Ueha-Ishibashi *et al.*, 2004a, 2004b; Westphal *et al.*, 2003), but the unique dependence of the developing nervous system on growth factors suggests that the neurotrophins and their receptors represent a possible target for thimerosal. There are a several studies suggesting that thimerosal may alter neurotrophin signaling, including binding of secondary messengers (Vanlingen *et al.*, 2001), microtubule assembly

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Calcium and Calmodulin Regulate Mercury-induced Phospholipase D Activation in Vascular Endothelial Cells

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Earlier, we reported that mercury, the environmental risk factor for cardiovascular diseases, activates vascular endothelial cell (EC) phospholipase D (PLD). Here, we report the novel and significant finding that calcium and calmodulin regulated mercury-induced PLD activation in bovine pulmonary artery ECs (BPAECs). Mercury (mercury chloride, 25 μM; thimerosal, 25 μM; methylmercury, 10 μM) significantly activated PLD in BPAECs. Calcium chelating agents and calcium depletion of the medium completely attenuated the mercury-induced PLD activation in ECs. Calmodulin inhibitors significantly attenuated mercury-induced PLD activation in BPAECs. Despite the absence of L-type calcium channels in ECs, nifedipine, nimodipine, and diltiazem significantly attenuated mercury-induced PLD activation and cytotoxicity in BPAECs. This study demonstrated the importance of calcium and calmodulin in the regulation of mercury-induced PLD activation and the protective action of L-type calcium channel blockers against mercury cytotoxicity in vascular ECs, suggesting mechanisms of mercury vasculotoxicity and mercury-induced cardiovascular diseases.

Keywords: calcium; calmodulin; L-type calcium channel blockers; lipid signaling; mercury; phospholipase D; phosphatidic acid; vascular endothelial cells

Mercury (Hg), a heavy metal belonging to the transition metal series of the periodic table, is an established environmental pollutant with known toxicity in humans. Mercury is widely recognized for its cytotoxicity, neurotoxicity, and immunotoxicity, and it appears to play no known physiological role.[1-3] Mercury usage in several devices causes accidental and occupational exposure to the metal among humans.[1] Inorganic mercury, in the form of chloride, is toxic to many organisms, including humans, and can readily undergo microbial biomethlyation to form the highly toxic organic form, methylmercury.[4] Methylmercury has been shown to cause hypertension in rats.[5] It has also been documented that methylmercury generates reactive oxygen species (ROS), leading to cellular oxidative stress.[6,7] Elemental mercury (Hg^0) was commonly used in dental practices for the greater part of the 20th century, and mercury vapor released from amalgam surfaces in the mouth is the predominant source of mercury exposure in the general population.[8] Persistent use of thimerosal (an organic mercurial)

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Mechanisms of Hg species induced toxicity in cultured human astrocytes: genotoxicity and DNA-damage response

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The toxicologically most relevant mercury (Hg) species for human exposure is methylmercury (MeHg). Thiomersal is a common preservative used in some vaccine formulations. The aim of this study is to get further mechanistic insight into the yet not fully understood neurotoxic modes of action of organic Hg species. Mercury species investigated include MeHgCl and thiomersal. Additionally $HgCl_2$ was studied, since in the brain mercuric Hg can be formed by dealkylation of the organic species. As a cellular system astrocytes were used. *In vivo* astrocytes provide the environment necessary for neuronal function. In the present study, cytotoxic effects of the respective mercuricals increased with rising alkylation level and correlated with their cellular bioavailability. Further experiments revealed for all species at subcytotoxic concentrations no induction of DNA strand breaks, whereas all species massively increased H_2O_2-induced DNA strand breaks. This co-genotoxic effect is likely due to a disturbance of the cellular DNA damage response. Thus, at nanomolar, sub-cytotoxic concentrations, all three mercury species strongly disturbed poly(ADP-ribosyl)ation, a signalling reaction induced by DNA strand breaks. Interestingly, the molecular mechanism behind this inhibition seems to be different for the species. Since chronic PARP-1 inhibition is also discussed to sacrifice neurogenesis and learning abilities, further experiments on neurons and *in vivo* studies could be helpful to clarify whether the inhibition of poly(ADP-ribosyl)ation contributes to organic Hg induced neurotoxicity.

Introduction

The three most exposure relevant chemical forms of mercury (Hg) are elemental Hg, mercuric Hg and organometallic compounds. Among these methylmercury is by far the most common in the environment and in the aquatic food-chain. In addition to the consumption of inorganic Hg contaminated food, inorganic Hg exposure might occur through medicinal products.[1] Inhaled elemental Hg vapour from dental amalgam is another source that is likely to increase internal Hg exposure.[2] Methylmercury exposure occurs nearly exclusively *via* fish and seafood, with generally 80–100% of total fish Hg being methylmercury.[2] In Hg polluted areas in China, methylmercury contaminated rice is a further possible contributor.[3] Non-dietary exposure to organic Hg might result from the application of thiomersal (sodium 2-ethylmercurothio-salicylate). Thiomersal is used as a preservative in multidose vials of some vaccines,[4] as well as in several cosmetic products and cleaning solutions for contact lenses.[5] Its antimicrobial effect is based on its decomposition in aqueous medium to thiosalicylic acid and the ethylmercury cation. Rapid hydrolysis of thiomersal in aqueous biological solution has been demonstrated before.[6]

In 2003, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) revised the PTWI for methylmercury to 1.6 μg kg^{-1} body weight (b.w.); developmental neurotoxicity was identified as the most sensitive toxicological endpoint. In 2006 JECFA confirmed this PTWI.[7] Based on the information that beneficial nutrients in fish may have confounded previous adverse outcomes in some of these studies, the European Food Safety Authority (EFSA) Scientific Panel on Contaminants in the Food Chain established in December 2012 a TWI for methylmercury of 1.3 μg kg^{-1} b.w. Moreover, the Panel concluded that high fish consumers, which might include pregnant women,

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EFFECT OF THIMEROSAL AND OTHER SULFHYDRYL REAGENTS ON CALCIUM PERMEABILITY IN THYMUS LYMPHOCYTES

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Abstract—We have studied the effects of thimerosal, a mercurial compound extensively used as a preservative, as well as other sulfhydryl reagents (e.g. *p*-hydroxymercurybenzoate, hydrogen peroxide, bromophenacyl bromide, and mercuric chloride) on Ca^{2+} homeostasis and the redox status of sulfhydryl groups in thymus lymphocytes. They all induced an increase in $[Ca^{2+}]_i$ which was blocked with dithiothreitol, suggesting that they act via the oxidation or blockade of sulfhydryl groups. $[Ca^{2+}]_i$ increase could be directly related to the effect of the different reagents on cellular protein sulfhydryl content. Experiments with ethidium bromide indicate that the observed rise in $[Ca^{2+}]_i$ was not due to a non-specific increase in membrane permeability. Thimerosal differs from the other agents studied in its oxidative properties, which is probably linked to the production of a potent reductor molecule, thiosalicylic acid, which may modulate its oxidative capacity.

Key words: Ca^{2+} homeostasis; thymus lymphocytes; thimerosal; sulfhydryl reagents

Thimerosal is used as a preservative in many pharmaceutical solutions and has also been reported to elicit the production of specific antibodies and cell-mediated immunity [1, 2]. These effects are related to its oxidative capacity [2]. The redox status of a cell is an important factor in the maintenance of general cellular homeostasis, in particular calcium homeostasis [3–7]. Essential sulfhydryl groups are present in a number of membrane-bound proteins related to Ca^{2+} permeability, and it is known that sulfhydryl reagents may alter the internal Ca^{2+} concentration [8–10]. We have previously described that thimerosal induces an increase in $[Ca^{2+}]_i$ in rat thymus lymphocytes [11], as well as in other cell preparations [12–19].

The effect of thimerosal on $[Ca^{2+}]_i$ homeostasis is complex, varying with dose and cell type [15–20]. In some cases, it has been proposed that thimerosal induces an increase in $[Ca^{2+}]_i$ by sensitizing the $InsP_3$ receptor, a phenomenon also observed with oxidized glutathione and *t*-butyl hydroperoxide at higher concentrations [18, 21]. In other preparations, thimerosal seems to open a pathway for Ca^{2+} entry from the extracellular side [11, 19].

The present study analyses the effects of thimerosal and other sulfhydryl reagents, including BPB, H_2O_2, $HgCl_2$ and pHMB,† on $[Ca^{2+}]_i$ in thymus lymphocytes. Although the compounds used differed in their mechanisms of action, their effects in all cases were abolished with DTT. We provide evidence that there is a relationship between the SH-blocking capacity and the potency of these agents in increasing $[Ca^{2+}]_i$. These effects are not a general consequence of cell damage since cell viability as evidenced by LDH release and ethidium bromide uptake was not affected by brief exposure to thiol reagents.

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† Abbreviations: AA, arachidonic acid; BPB, bromophenacyl bromide; $[Ca^{2+}]_i$, internal calcium concentration; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; p-HMB, *p*-hydroxymercuribenzoate; LDH, lactate dehydrogenase; 2-ME, 2-mercaptoethanol; PHA, phytohaemagglutinin; PLA_2, phospholipase A_2; SH, sulfhydryl; TNB, 2-nitro-5-thiobenzoic acid.

MATERIALS AND METHODS

Cell isolation. Thymocytes were prepared from 6-week-old Wistar rats of either sex as previously described [11], and kept in a standard saline composed of (in mmol/L): 125 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 1.5 NaH_2PO_4, 10 glucose and 25 Hepes, pH 7.4. Standard saline was used in all experiments unless otherwise indicated.

Measurement of $[Ca^{2+}]$ *concentration.* Loading cells with Fura-2/AM and measurement of $[Ca^{2+}]_i$ were performed as previously described [11]. For measurement of $[Ca^{2+}]_i$, thymocytes were suspended in a thermostatically controlled and magnetically stirred fluorimeter cuvette (Perkin-Elmer LS5) at a concentration of $1–2 \times 10^7$ cells/mL. Since phosphate, when complexed with manganese, increases autofluorescence at the excitation wavelength of Fura-2, free phosphate was omitted from the reaction mixture in all experiments. To prevent Fura-2 leakage from the cells we used 2.5 mM probenecid. Fura-2 leakage was determined in the calibration cuvettes by the decrease in fluorescence after the addition of 100 μM $MnCl_2$. Only cell preparations with less than 1–2% fluorescence decrease (100 is the arbitrary unit for fluorescence in the presence of digitonin) were used for the experiments.

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Short communication

Dose-response analysis indicating time-dependent neurotoxicity caused by organic and inorganic mercury—Implications for toxic effects in the developing brain

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ABSTRACT

A latency period preceding neurotoxicity is a common characteristic in the dose-response relationship induced by organic mercury. Latency periods have typically been observed with genotoxicants in carcinogenesis, with cancer being manifested a long time after the initiating event. These observations indicate that even a very small dose may cause extensive adverse effects later in life, so the toxicity of the genotoxic compound is dose and time-dependent. In children, methylmercury exposure during pregnancy (in utero) has been associated with delays in reaching developmental milestones (e.g., age at first walking) and decreases in intelligence, increasing in severity with increasing exposure. Ethylmercury exposure from thimerosal in some vaccines has been associated, in some studies, with autism and other neurological disorders in children. In this paper, we have examined whether dose-response data from *in vitro* and *in vivo* organic mercury toxicity studies fit the Druckrey-Küpfmüller equation $c \cdot t^n = \text{constant}$ (c = exposure concentration, t = latency period), first established for genotoxic carcinogens, and whether or not irreversible effects are enhanced by time of exposure ($n \geq 1$), or else toxic effects are dose-dependent while time has only minor influence on the adverse outcome ($n < 1$). The mode of action underlying time-dependent toxicity is irreversible binding to critical receptors causing adverse and cumulative effects. The results indicate that the Druckrey-Küpfmüller equation describes well the dose-response characteristics of organic mercury induced neurotoxic effects. This amounts to a paradigm shift in chemical risk assessment of mercurial compounds and highlights that it is vital to perform toxicity testing geared to investigate time-dependent effects.

1. Introduction

Organic mercury induced neurotoxicity has typically been observed after a preceding latency period. Even severe and fatal ethylmercury intoxications in humans featured a latency period between cessation of exposure and onset of first symptoms of 10 days to 7 weeks (Cinca et al., 1980; Magos, 2001). For methylmercury, latencies in intoxications in Iraq and Japan ranged from weeks to more than a year (Bakir et al., 1973; National Research Council, 2000; Weiss et al., 2002) and effects were proceeding even after exposure had ended 20–30 years before (Rice and Barone, 2000). When monkeys were exposed to low levels of methylmercury during their developmental phase, neurotoxicity appeared after several years (Rice, 1996). Latency periods have typically been observed with genotoxicants in carcinogenesis, with cancer being manifested a long time after the initiating event. These observations indicate that even a very small dose may cause extensive adverse effects later in life, so the toxicity of the genotoxic compound is dose and time-dependent.

Methylmercury is widely distributed throughout the environment, particularly in estuarine and marine sediments (Bryan and Langston, 1992; Compeau and Bartha, 1985; Morel et al., 1998) and accumulates in fish and birds (Greichus et al., 1973; Harris et al., 2007; Henny et al., 2005; Houserová et al., 2007; Lam et al., 2005; Polak-Juszczak, 2012; Wren, 1986). Therefore, people are likely to be continuously exposed to small amounts of methylmercury through consumption of contaminated food (Chan et al., 2010; Lin et al., 2012). Ethylmercury is used as preservative in vaccines that may be administered to pregnant women. Toxicokinetic evidence confirms that alkyl mercury compounds cross the placental barrier (Aschner and Clarkson, 1988; Bridges and Zalups, 2005; Dórea,

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Determination of Methylmercury, Ethylmercury, and Inorganic Mercury in Mouse Tissues, Following Administration of Thimerosal, by Species-Specific Isotope Dilution GC−Inductively Coupled Plasma-MS

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Isotopically enriched HgO standards were used to synthesize $CH_3{}^{200}Hg^+$ and $C_2H_5{}^{199}Hg^+$ using Grignard reagents. These species were employed for isotope dilution GC−ICPMS to study uptake and biotransformation of ethylmercury in mice treated with thimerosal, (sodium ethylmercurithiosalicylate) 10 mg L^{-1} in drinking water ad libitum for 1, 2.5, 6, or 14 days. Prior to analysis, samples were spiked with aqueous solutions of $CH_3{}^{200}Hg^+$, $C_2H_5{}^{199}Hg^+$, and $^{201}Hg^{2+}$ and then digested in 20% tetramethylammonium hydroxide and extracted at pH 9 with DDTC/toluene. Extracted mercury species were reacted with butylmagnesium chloride to form butylated derivatives. Absolute detection limits for CH_3Hg^+, $C_2H_5Hg^+$, and Hg^{2+} were 0.4, 0.2, and 0.6 pg on the basis of 3σ of five separate blanks. Up to 9% of the $C_2H_5Hg^+$ was decomposed to Hg^{2+} during sample preparation, and it is therefore crucial to use a species-specific internal standard when determining ethylmercury. No demethylation, methylation, or ethylation during sample preparation was detected. The ethylmercury component of thimerosal was rapidly taken up in the organs of the mice (kidney, liver, and mesenterial lymph nodes), and concentrations of $C_2H_5Hg^+$ as well as Hg^{2+} increased over the 14 days of thimerosal treatment. This shows that $C_2H_5Hg^+$ in mice to a large degree is degraded to Hg^{2+}. Increased concentrations of CH_3Hg^+ were also observed, which was found to be due to impurities in the thimerosal.

The difference in toxicity of various mercury species[1] makes it important to determine the atomic and molecular forms of mercury in tissues after acute and chronic exposure. Since organic mercury compounds might be transformed to Hg^{2+} in the tissues, it is of interest to study the temporal aspects of transport and transformation of various mercury species.

Organic mercury compounds, primarily CH_3Hg^+ and $C_2H_5Hg^+$, were introduced as agricultural fungicides at the beginning of the 20th century, but after a series of accidental mercury poisonings with fatal outcome[2] and evidence of environmental hazards,[3] alkylmercury compounds were discontinued for agricultural use. Paradoxically, until recently, $C_2H_5Hg^+$ in the form of thimerosal (sodium ethylmercurithiosalicylate) was added (0.003−0.01%) to several medical preparations for antimicrobial purposes.[4] Then in 1999, the U.S. Public Health Service (USPHS) and the American Academy of Pediatrics (AAP) issued a joint statement[5] in which they identified thimerosal as a widespread source of organic mercury exposure in infants/small children and recommended that it should be reduced or eliminated from childhood vaccines. It has been estimated that an infant might be exposed to ∼200 μg of Hg (as $C_2H_5Hg^+$) during the first 6 months of life through vaccinations.[4] The effect of childhood $C_2H_5Hg^+$ exposure has not been systematically studied, but the qualitative effect is thought to be similar to that of methylmercury,[4] which in sufficient doses causes widespread damage to the developing central nervous system.[6] Recently, the Immunization Safety Review Committee of the U.S. Institute of Medicine stated that the hypothesis[7,8] that exposure to thimerosal-containing vaccines is associated with

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Chemical compounds that target thiol-disulfide groups on mononuclear phagocytes inhibit immune mediated phagocytosis of red blood cells

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BACKGROUND: Patients having immune cytopenias produce antibodies that target hematopoietic cells resulting in their phagocytosis and intracellular destruction. Early reports suggested that phagocytosis could be inhibited by interfering with membrane thiol (SH) groups on phagocytes. Thus, whether chemical compounds that interact with SH or disulfide (SS) groups on mononuclear phagocytes can inhibit phagocytosis of antibody-coated cells was examined.

STUDY DESIGN AND METHODS: A monocyte monolayer assay (MMA), which examines the in vitro monocyte-macrophage (Mϕ) interaction with anti-Rh(D)-coated red cells (RBCs), was used to study the ability of different SH and SS chemicals to inhibit the Fc receptor–mediated phagocytosis of sensitized RBCs. The compounds examined included thimerosal, dithiothreitol (DTT), pentane-1-thiol, and two recently described SH and two SS chemicals that have been synthesized.

RESULTS: All compounds were found to be able to inhibit phagocytosis to varying degrees correlating to the structure of the molecule. In general, those compounds that interact with free SH groups to inhibit phagocytosis were found better than SH-containing compounds that interact with SSs. Thimerosal and p-nitrophenyl methyl disulfide were the most effective compounds inhibiting phagocytosis. Both chemicals showed greater than 50 percent inhibition at concentrations as low as 10^{-9} mol per L. DTT was the least effective compound tested. Only thimerosal showed significant toxicity, as determined by decreased cell viability and increased apoptosis, but only at concentrations of 10^{-8} mol per L. The effect of chemical treatment was on attachment rather than on phagocytosis itself. Fcγ receptor–independent endocytosis was not affected by the chemical treatment.

CONCLUSION: These studies indicate that pharmacologic strategies that target SH groups on mononuclear phagocytes may have future efficacy for the treatment of immune cytopenias.

Immune cytopenias are pathologic conditions where patients make antibodies to specific hematopoietic cells in the blood.[1-4] In these conditions, the cells become coated with antibodies and are subsequently recognized by the Fc-γ receptors (FcγRs) on the mononuclear phagocyte membrane.[4] Current therapies for the treatment of severe cases of immune cytopenias include splenectomy and administration of steroids or immunoglobulins.[5-9] Intravenous immunoglobulin (IVIG) and RhIG are both used with varied success to treat immune cytopenias.[5,6,10] Both IVIG and anti-D, however, are a limited resource[11] owing to their acquisition from human donations. Treatment with IVIG is more costly than with anti-D[12] primarily because of the amount of IVIG required for effective therapy; the usual induction dose is 2000 mg IVIG per kilogram of body weight, which may be followed

ABBREVIATIONS: F-B = p-toluenesulfonylmethyl mercaptan; FcγR(s) = Fc-γ receptor(s); G-B = p-nitrophenyl methyl disulfide; Mϕ = monocyte macrophage; MMA = monocyte monolayer assay; SH = thiol; SS(s) = disulfide(s).

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NeuroToxicology

Predicted Mercury Concentrations in Hair From Infant Immunizations: Cause for Concern☆

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Abstract

Mercury (Hg) is considered one of the worlds most toxic metals. Current thinking suggests that exposure to mercury occurs primarily from seafood contamination and rare catastrophic events. Recently, another common source of exposure has been identified. Thimerosal (TMS), a preservative found in many infant vaccines, contains 49.6% ethyl mercury (EtHg) by weight and typically contributes 25 μg. of EtHg per dose of infant vaccine. As part of an ongoing review, the Food and Drug Administration (FDA) announced in 1999 that infants who received multiple TMS-preserved vaccines may have been exposed to cumulative Hg in excess of Federal safety guidelines. According to the centers for disease control (CDC) recommended immunization schedule, infants may have been exposed to 12.5 μg Hg at birth, 62.5 μg EtHg at 2 months, 50 μg EtHg at 4 months, 62.5 μg EtHg at 6 months, and 50 μg EtHg at approximately 18 months, for a total of 237.5 μg EtHg during the first 18 months of life, if all TMS-containing vaccines were administered. Neurobehavioral alterations, especially to the more susceptible fetus and infant, are known to occur after relatively low dose exposures to organic mercury compounds. In effort, to further elucidate the levels of ethyl mercury resulting from exposure to vaccinal TMS, we estimated hair Hg concentrations expected to result from the recommended CDC schedule utilizing a one compartment pharmacokinetic model. This model was developed to predict hair concentrations from acute exposure to methymercury (MeHg) in fish. Modeled hair Hg concentrations in infants exposed to vaccinal TMS are in excess of the Environmental Protection Agency (EPA) safety guidelines of 1 ppm for up to 365 days, with several peak concentrations within this period. More sensitive individuals and those with additional sources of exposure would have higher Hg concentrations. Given that exposure to low levels of mercury during critical stages of development has been associated with neurological disorders in children, including ADD, learning difficulties, and speech delays, the predicted hair Hg concentration resulting from childhood immunizations is cause for concern. Based on these findings, the impact which vaccinal mercury has had on the health of American children warrants further investigation. © *2001 Published by Elsevier Science Inc.*

Keywords: Mercury; Vaccine; Neurotoxicity; Thimerosal; Learning disabilities

INTRODUCTION

Mercury is a potent human toxicant that has long been the source of serious health problems. Toxicologic manifestations of mercury exposure have become known through hundreds of years of medicinal applications, industrial uses, and environmental tragedies. After exposure to mercury, deposition has been found in all body tissue. Therefore, it is not surprising that the clinical manifestations of mercury toxicity involve multiple organ systems with variable features and intensity. These manifestations vary by the route of exposure, the chemical form of mercury involved, the acuity of the intoxication, and the age at exposure (Goldfrank et al., 1998). Also, a mercury dose given acutely may produce toxic effects whereas the same dose distributed over a period of time may give no evidence of toxicity (Koos and Longo, 1976).

☆Modeled hair mercury concentrations arising from exposure to mercury-containing infant vaccines show elevations in excess of Federal safety guidelines for extended periods and with several peaks. Elevations over guidelines occur during critical stages of infant development. Predicted Hg levels are cause for concern, and further research is warranted.

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IN VITRO SYSTEMS

A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors

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Abstract Test systems to identify developmental toxicants are urgently needed. A combination of human stem cell technology and transcriptome analysis was to provide a proof of concept that toxicants with a related mode of action can be identified and grouped for read-across. We chose a test system of developmental toxicity, related to the generation of neuroectoderm from pluripotent stem cells (UKN1), and exposed cells for 6 days to the histone deacetylase inhibitors (HDACi) valproic acid, trichostatin A, vorinostat, belinostat, panobinostat and entinostat. To provide insight into their toxic action, we identified HDACi consensus genes, assigned them to superordinate biological processes and mapped them to a human transcription factor network constructed from hundreds of transcriptome data sets. We also tested a heterogeneous group of 'mercurials' (methylmercury, thimerosal, mercury(II)chloride, mercury(II)bromide, 4-chloromercuribenzoic acid, phenylmercuric acid). Microarray data were compared at the highest non-cytotoxic concentration for all 12 toxicants. A support vector machine (SVM)-based classifier predicted all HDACi correctly. For validation, the classifier was applied to legacy data sets of HDACi, and for each exposure situation, the SVM predictions correlated with the developmental toxicity. Finally, optimization of the classifier based on 100 probe sets showed that eight genes (F2RL2, TFAP2B, EDNRA, FOXD3, SIX3, MT1E, ETS1 and LHX2) are sufficient to separate HDACi from mercurials. Our data demonstrate how human stem cells and transcriptome analysis can be combined for mechanistic grouping and prediction of toxicants. Extension of this concept to mechanisms beyond HDACi would allow prediction of human developmental toxicity hazard of unknown compounds with the UKN1 test system.

Keywords Hazard assessment · Neuronal development · Alternative testing · Cytotoxicity · Transcriptomics · Developing central nervous system

Eugen Rempel and Lisa Hoelting shared first authorship.

Jörg Rahnenführer, Jan G. Hengstler and Marcel Leist shared senior authorship.

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Organic mercury compounds: human exposure and its relevance to public health

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Humans may be exposed to organic forms of mercury by either inhalation, oral, or dermal routes, and the effects of such exposure depend upon both the type of mercury to which exposed and the magnitude of the exposure. In general, the effects of exposure to organic mercury are primarily neurologic, while a host of other organ systems may also be involved, including gastrointestinal, respiratory, hepatic, immune, dermal, and renal. While the primary source of exposure to organic mercury for most populations is the consumption of methylmercury-contaminated fish and shellfish, there are a number of other organomercurials to which humans might be exposed. The antibacterial and antifungal properties of organomercurials have resulted in their long use as topical disinfectants (thimerosal and merbromin) and preservatives in medical preparations (thimerosal) and grain products (both methyl and ethyl mercurials). Phenylmercury has been used in the past in paints, and dialkyl mercurials are still used in some industrial processes and in the calibration of certain analytical laboratory equipment. The effects of exposure to different organic mercurials by different routes of exposure are summarized in this article. *Toxicology and Industrial Health* 2002; **18:** 109–160.

Key words: *ethylmercury; mercury; methylmercury; organomercurials; phenylmercury; thimerosal*

Introduction

Mercury is a naturally occurring element in the earth's crust. Over geological time, it has been distributed throughout the environment by natural processes, such as volcanic activity, fires, movement of rivers, lakes, and streams, oceanic upwelling, and biological processes. Since the advent of the industrial revolution over 200 years ago, however, anthropogenic sources have become a significant contributor to the environmental distribution of mercury and its compounds.

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In the environment, elemental mercury can combine with chlorine, sulfur, phosphorous, and other elements to form inorganic compounds. Primarily through the action of micro-organisms, inorganic mercury can be combined with carbon to form organic mercury compounds, of which methylmercury is the most abundant. In surface waters, it is rapidly accumulated by aquatic organisms, where it biomagnifies as it ascends the food chain.

In addition to methylmercury, there are a number of other organomercurials to which humans might be exposed. The antibacterial and antifungal properties of organomercurials have resulted in their long use as topical disinfectants (thimerosal and

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Abstract There are a number of mechanisms by which alkylmercury compounds cause toxic action in the body. Collectively, published studies reveal that there are some similarities between the mechanisms of the toxic action of the mono-alkyl mercury compounds methylmercury (MeHg) and ethylmercury (EtHg). This paper represents a summary of some of the studies regarding these mechanisms of action in order to facilitate the understanding of the many varied effects of alkylmercurials in the human body. The similarities in mechanisms of toxicity for MeHg and EtHg are presented and compared. The difference in manifested toxicity of MeHg and EtHg are likely the result of the differences in exposure, metabolism, and elimination from the body, rather than differences in mechanisms of action between the two.

Keywords Arachidonic acid • Calcium homeostasis • Cell cycle/division • Ethylmercury • Glial cells • Glutamate • Glutamine • Glutathione (GSH) • Leukotriene synthesis mechanism of toxicity • Membrane permeability/integrity • Methylmercury • Mitochondria • Neurotransmitter release nitric oxide • Oxidative stress • Reactive oxygen species • Receptor binding • ROS • Thimerosal

1 Preface

The alkyl mercury compounds methylmercury (MeHg) and ethylmercury (EtHg) have been shown to be toxic to humans and non-human animals as well (Driscoll et al. 2013). Both of those compounds have similar chemical properties, and both have been shown to disrupt the normal function of the CNS in a variety of animal species.

In mass poisonings from the consumption of MeHg-contaminated seafood in Japan (Kutsuna 1968) and MeHg-treated grain in Iraq (Bakir et al. 1973), frank developmental/birth effects and/or severe brain damage were observed in the children of some mothers who consumed large quantities of mercury-contaminated fish, bread, or grain products during pregnancy. In addition, a variety of neurologic effects were reported in both the 1968 and 1973 papers. Among these effects were deficits in cognitive and motor function. Some of the many specific effects observed were distal paresthesias, ataxia, unsteady gait, muscle weakness, impairment of the senses, irritability, memory loss, insomnia, and confusion. Similar neurologic disorders were seen in Iraq in 1956 and 1960, when food containing flour made from grain treated with EtHg *p*-toluene sulfonanilide was consumed (Bakir et al. 1973; Jalili and Abbasi 1961). In another reported incident (Zhang 1984), 41 individuals were poisoned by eating rice that had been treated with a grain disinfectant containing 2–2.5 % $EtHgCl_2$. Many of the symptoms and clinical signs were similar to those experienced in the ethyl- and methylmercury poisonings in Japan and Iraq. And while both of the alkyl compounds primarily effect the central nervous system, the rapid metabolism of ethylmercury to inorganic mercury may lead to kidney damage (Clarkson and Magos 2006), especially at higher amounts and with longer periods of exposure.

BRIEF REPORT

ARE MERCURY AMALGAM FILLINGS SAFE FOR CHILDREN? AN EVALUATION OF RECENT RESEARCH RESULTS

Dorena Rode, PhD

Two recent clinical trials on the safety of amalgam fillings in children found no evidence of harmful effects from mercury-containing dental fillings after following children for 5-7 years. This review suggests the studies' results are limited by (1) sample sizes that were too small to allow detection of genetic variations in mercury toxicity at a rate of 1 in 100 or lower, (2) a lack of control for other sources of mercury, and (3) a population that may have been skewed by excluding children with autism during a time when autism was escalating due, in part, to increased frequency of thimerosal-containing vaccine use.

Dorena Rode, PhD, is director of Research and Development at EcoNugenics Inc, Santa Rosa, Calif.

Two thoughtfully designed and implemented clinical trials on the safety of amalgam fillings in children were published in *JAMA* in April. Neither of these National Institutes of Health (NIH)–funded studies found evidence of harmful effects from mercury-containing dental fillings after following children for 5-7 years. This paper reviews the studies and discusses their limitations in defining the safety of amalgams in children.

UNITED STATES–BASED STUDY

In the United States study, 267 children receiving amalgam fillings and 267 children receiving resin composite fillings were followed for 5 years.[1] Eligible participants were children 6-10 years old with no prior amalgam fillings and no physician-diagnosed psychological, behavioral, neurological, immunosuppressive, or renal disease. This study was designed to detect an IQ difference of 3 points between children with caries filled with amalgam versus composite and also to explore potential differences in visuomotor ability, memory, and kidney function. There were no significant differences between the groups for any parameter at any time point. In addition, the 2 groups were monitored for development of new health conditions during the 5-year follow-up. The 2 groups reported similar incidences of new health conditions, including allergy, asthma, migraines, skin disorders, respiratory disorders, psychological disorders, and gastrointestinal disorders, among others. The researchers reported that children with amalgams had slight (0.9 µg/g creatinine) but significant increases in urinary mercury compared to controls (0.6 µg/g creatinine). There was no difference in hair mercury content between the 2 groups.

LISBON, PORTUGAL–BASED STUDY

In the Portuguese study, 253 children receiving amalgam fillings and 252 children receiving resin composite fillings were followed for 7 years.[2] Eligible participants were children 8-10 years old with no prior amalgam fillings, urinary mercury below 10 µg/L, blood lead lower than 15 µg/L, IQ >67, and no interfering health conditions. This study also assessed memory, attention, visuomotor function, kidney function, and nerve conduction velocity. Endpoints were assessed annually, and there were no differences in any parameters at any time point. In this study, urinary mercury in the amalgam group was 1.5 µg/g creatinine greater than the composite group during the first 3 years and declined to an average 1.0 µg/g in later years, with no significant difference at year 7.

MERCURY TOXICITY

Amalgams are made of approximately 50% mercury. Mercury is a known neurotoxin. Exposure resulting in urinary levels of 50 to 200 µg/L is associated with neurobehavioral defects, such as reduced metal capability, loss of fine motor coordination, mood alterations, and insomnia.[3] There is some evidence that urinary concentrations of mercury as low as 4 µg/L are associated with mood changes,[4] but in general, the effect of low-level mercury exposure is not well defined. Because of this deficiency, the World Health Organization has requested that researchers focus on investigating threshold effects at levels below 25 µg/L, as mean urinary levels in the general population are 3.1 or 9.0 µg/L.[4] These 2 amalgam studies should have provided useful information about low-level mercury exposure, but the similarity in urinary mercury excretion between the controls and the group receiving amalgams actually suggests similar mercury exposure.

STUDY LIMITATIONS

Despite meaningful endpoints reflecting parameters

impacted by mercury, the studies failed to uncover any evidence of mercury toxicity from amalgams. The research has several key limitations, however. The researchers themselves admit, "This study was not designed to detect whether a very small fraction of children may have genetic predispositions to sequester elemental mercury at an extraordinarily high rate, or have rare allergic or other kinds of adverse reactions to elemental mercury."[2(p1791)] The small sample size of the studies does not allow for the detection of adverse effects if they occurred at a rate lower than 1 in 100.

Current research suggests that genetic variability may result in children that are susceptible to small amounts of mercury. This may be due to an inability to detoxify and eliminate mercury, resulting in manifestations of neurotoxicty and immune dysregulation as might be expected from higher mercury exposure. Indeed, recent research in human adults with occupational mercury exposure has demonstrated a correlation between neurobehavioral dysfunction from mercury exposure and genetic variants of an enzyme involved in heme synthesis as well as a neurotrophic protein.[5,6] Genetic variation of other enzymes also is suspected to contribute to mercury sensitivity, and multiple mutations may be a prerequisite to seeing clinical manifestations.

SKEWED STUDY POPULATION?

The slight differences in urinary mercury between the 2 groups in both studies suggest that exposure from the fillings may have been insignificant compared to exposure from vaccines and diet. Neither study reports on or controls for other sources of mercury, and the studies were conducted in a time period when immunization with thimerosal-containing vaccines was routine. It is possible that the recruitment of healthy school-age children may have excluded children with any and all genetic susceptibility to mercury. Research suggests that toxic metals, and mercury specifically, may play a role in autism.[7,8] Indeed, the phasing out of thimerosal-containing childhood vaccines in California has resulted in a decrease in the incidence of autism.[9] Given that autistic spectrum disorder (ASD) is typically diagnosed in the first 5 years of life and during the time period of the studies there was a 6-fold increase in ASD diagnoses,[10] one wonders if the children in the study may not have been truly representative of the general population. Considering the Centers for Disease Control and Prevention's estimates of ASD prevalence as high as 1 in 166 individuals,[11] the study may have ended up with a skewed population of only the fittest.

CONCLUSION

The 2 published amalgam studies reported unexpected results. However, given the extremely small differences in the urinary mercury levels between the children with amalgams and the composite controls, the studies may be lacking an adequate control group. Future studies aimed at determining the health risk of amalgams will need to ensure all other sources of mercury are removed and previous significant exposures from diet and vaccines are controlled for. In addition, research is needed to determine the prevalence of genetic predisposition to mercury toxicity and the long-term health effects of decades of exposure to mercury from amalgams.

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INORGANIC COMPOUNDS

Identification and distribution of mercury species in rat tissues following administration of thimerosal or methylmercury

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Abstract Methylmercury (Met-Hg) is one the most toxic forms of Hg, with a considerable range of harmful effects on humans. Sodium ethyl mercury thiosalicylate, thimerosal (TM) is an ethylmercury (Et-Hg)-containing preservative that has been used in manufacturing vaccines in many countries. Whereas the behavior of Met-Hg in humans is relatively well known, that of ethylmercury (Et-Hg) is poorly understood. The present study describes the distribution of mercury as (-methyl, -ethyl and inorganic mercury) in rat tissues (brain, heart, kidney and liver) and blood following administration of TM or Met-Hg. Animals received one dose/day of Met-Hg or TM by gavage (0.5 mg Hg/kg). Blood samples were collected after 6, 12, 24, 48, 96 and 120 h of exposure. After 5 days, the animals were killed, and their tissues were collected. Total blood mercury (THg) levels were determined by ICP-MS, and methylmercury (Met-Hg), ethylmercury (Et-Hg) and inorganic mercury (Ino-Hg) levels were determined by speciation analysis with LC-ICP-MS. Mercury remains longer in the blood of rats treated with Met-Hg compared to that of TM-exposed rats. Moreover, after 48 h of the TM treatment, most of the Hg found in blood was inorganic. Of the total mercury found in the brain after TM exposure, 63% was in the form of Ino-Hg, with 13.5% as Et-Hg and 23.7% as Met-Hg. In general, mercury in tissues and blood following TM treatment was predominantly found as Ino-Hg, but a considerable amount of Et-Hg was also found in the liver and brain. Taken together, our data demonstrated that the toxicokinetics of TM is completely different from that of Met-Hg. Thus, Met-Hg is not an appropriate reference for assessing the risk from exposure to TM-derived Hg. It also adds new data for further studies in the evaluation of TM toxicity.

Keywords Mercury · Distribution · Methylmercury · Ethylmercury · Inorganic mercury · Speciation analysis · Tissues · Toxicity · Thimerosal

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Introduction

Methylmercury (Met-Hg) is one the most toxic forms of Hg and the most common form of mercury exposure. A considerable range of harmful effects on humans has been identified (Grotto et al. 2009a, b; Mori et al. 2007; Yamamoto and Shima 2009). On other hand, sodium ethyl mercury thiosalicylate (thimerosal) is an ethylmercury (Et-Hg)-containing preservative that has been used for over 60 years as an antimicrobial agent in vaccines to prevent contamination (Tan and Parkin 2000). In spite of the huge information about methylmercury metabolism, little is known about thimerosal (ethylmercury) disposition in mammals.

The use of thimerosal (TM) has probably prevented the death or illness of countless infants by reducing the risk of contamination from open multidose vials, for example. However, some experimental and epidemiological studies have shown associations between increased Hg exposure from TM-containing vaccines and toxic effects (Berman et al. 2008; Geier and Geier 2006; Westphal et al. 2003). For this reason, this compound has been removed from many childhood vaccines in several countries including United States. However, TM-preserved vaccines are still in use around the world in situations where the advantages of

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Toxicological effects of thiomersal and ethylmercury: Inhibition of the thioredoxin system and NADP$^+$-dependent dehydrogenases of the pentose phosphate pathway

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ABSTRACT

Mercury (Hg) is a strong toxicant affecting mainly the central nervous, renal, cardiovascular and immune systems. Thiomersal (TM) is still in use in medical practice as a topical antiseptic and as a preservative in multiple dose vaccines, routinely given to young children in some developing countries, while other forms of mercury such as methylmercury represent an environmental and food hazard. The aim of the present study was to determine the effects of thiomersal (TM) and its breakdown product ethylmercury (EtHg) on the thioredoxin system and NADP$^+$-dependent dehydrogenases of the pentose phosphate pathway. Results show that TM and EtHg inhibited the thioredoxin system enzymes in purified suspensions, being EtHg comparable to methylmercury (MeHg). Also, treatment of neuroblastoma and liver cells with TM or EtHg decreased cell viability (GI_{50}: 1.5 to 20 μM) and caused a significant ($p < 0.05$) decrease in the overall activities of thioredoxin (Trx) and thioredoxin reductase (TrxR) in a concentration- and time-dependent manner in cell lysates. Compared to control, the activities of Trx and TrxR in neuroblastoma cells after EtHg incubation were reduced up to 60% and 80% respectively, whereas in hepatoma cells the reduction was almost 100%. In addition, the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also significantly inhibited by all mercurials, with inhibition intensity of $Hg^{2+} > MeHg \approx EtHg > TM$ ($p < 0.05$). Cell incubation with sodium selenite alleviated the inhibitory effects on TrxR and glucose-6-phosphate dehydrogenase. Thus, the molecular mechanism of toxicity of TM and especially of its metabolite EtHg encompasses the blockage of the electrons from NADPH *via* the thioredoxin system.

Introduction

Mercurial compounds have shown a wide range of toxicological effects on human beings, involving especially the central nervous system, causing damage to the brain, but also to the kidneys, the cardiovascular and immune systems (Clarkson et al., 2003; Dórea et al., 2013). Exposure to mercurial compounds such as methylmercury (MeHg) and mercuric mercury (Hg^{2+}) at levels above the toxicity threshold occurs either by regular fish consumption or occupational contact, respectively, and represents a major concern in toxicology (Clarkson et al., 2003; Carvalho et al., 2008a; Nunes et al., 2014). Not less important is mercury exposure in dental practice for both dentists and patients due to the use of dental amalgam fillings that release mercury vapour (Clarkson et al., 2003). Even though the use of mercury compounds such as thiomersal (TM) in medicines and antiseptics is decreasing it is still used as a preservative in some formulas, namely in vaccines (Sykes et al., 2014).

Although mercurial compounds are not new toxicants, there is a significant lack of knowledge about their molecular mechanisms of toxicity, especially about TM and its breakdown product ethylmercury (EtHg). TM, a mercury derivative composed of EtHg and thiosalicylic acid has been widely used as a preservative in vaccines, dermatological (topic) and ocular preparations. Indeed, vaccines with TM are the main route of mercury exposure in clinics (Bigham and Copes, 2005) and while children in most of the developed countries receive normally TM-free vaccines, children in developing countries may receive several doses of different

Abbreviations: 6PGDH, 6-phosphogluconate dehydrogenase; EtHg, ethylmercury; G6PDH, glucose-6-phosphate dehydrogenase; Hg^{2+}, mercuric mercury; MeHg, methylmercury; Se^{2-}, selenide; SeO_3^{2-}/Se (IV), selenite; Se, selenium; TCV, thiomersal-containing vaccines; TM, thiomersal; Trx, thioredoxin; TrxR, thioredoxin reductase.

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Mercury toxicity following merthiolate ear irrigations

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AQUEOUS MERTHIOLATE, an aqueous solution containing 0.1% thimerosal and 0.14% sodium borate, has been recommended as a treatment of purulent otitis media with spontaneous perforations, after insertion of tympanostomy tubes, for external otitis, and as an irrigant during mastoid surgery.[1,2] Although aqueous merthiolate has been used for years as a topical antiseptic, a recent review of its use by the Food and Drug Administration resulted in its classification as "less than effective."[3] Furthermore, two of the ingredients (thimerosal and borate) in merthiolate are toxic if absorbed or injected.

We describe mercury toxicity in a child after ear irrigations with aqueous merthiolate for 1 month.

CASE REPORT

An 18-month-old white infant girl was admitted with a diagnosis of chronic otitis media, ataxia, and irritability. One year previously, bilateral tympanostomy tubes had been inserted. Six weeks prior to admission, she developed purulent otitis media refractory to antibiotic therapy (amoxicillin, trimethoprim/sulfamethoxazole, then erythromycin/sulfisoxazole). One month prior to admission, daily ear irrigations with 1 oz aqueous merthiolate were prescribed. The frequency of irrigations was increased to twice daily 3 weeks prior to admission. A total of 1.2 L merthiolate was used over the 4 weeks.

Admission findings included a history of staring spells, ataxia, unprovoked screaming episodes associated with opisthotonic posturing, hand tremors, inability to feed herself, and vomiting. Her past medical history, including exposure to other toxins, was unremarkable. Growth and development had been appropriate for age. Initial physical examination showed only a small, irritable child who was difficult to console, with a left-sided draining otitis media and marked ataxia. Rectal temperature was 38.8° C, pulse 164, respirations 30, BP 108/70. Height was 76 cm (5% for age), and weight 9.42 kg (20%).

Blood and CSF cultures, viral serologic findings (including Epstein-Barr virus and hepatitis), CT scan, and mastoid films were all normal. The first EEG showed generalized slowing. By day 4 of admission the child was lethargic, and on day 5 she developed metabolic acidosis, dehydration, and hyperglycemia. Stage II coma and rotary nystagmus were evident by day 7, and on day 10 she required tracheal intubation and mechanical ventilation. The EEG on day 7 was even slower and more abnormal, with increased delta activity, and by day 10 showed burst suppression. The SGOT activity at that time was 1789 U/L (normal 8 to U/L), SGPT 1807 U/L (nl 5 to 35 U/L), prothrombin time 20.9 sec (n 10.4 to 12.5 sec), and activated PTT 190.3 sec (nl 26.4 to 40.0 sec). Serum lactate concentration was 24.7 mg/dl (nl 5 to 20 mg/dl), and pyruvate 0.6 mg/dl (nl 0.3 to 0.9 mg/dl). The urine showed a generalized aminoaciduria.

The patient developed persistent metabolic acidosis, with a large anion gap, hyperkalemia, renal and hepatic failure, hypertension, and congestive heart failure. She later developed a scaled skin picture, and culture proved staphylococcal sepsis. A toxicology consultation was obtained, and the history of previous exposure

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Autism: Transient *in utero* hypothyroxinemia related to maternal flavonoid ingestion during pregnancy and to other environmental antithyroid agents

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Available online 24 July 2007

Abstract

The incidence and prevalence of autism have increased during the past two decades. Despite comprehensive genetic studies the cause of autism remains unknown. This review emphasizes the potential importance of environmental factors in its causation. Alterations of cortical neuronal migration and cerebellar Purkinje cells have been observed in autism. Neuronal migration, *via* reelin regulation, requires triiodothyronine (T3) produced by deiodination of thyroxine (T4) by fetal brain deiodinases. Experimental animal models have shown that transient intrauterine deficits of thyroid hormones (as brief as 3 days) result in permanent alterations of cerebral cortical architecture reminiscent of those observed in brains of patients with autism. I postulate that early maternal hypothyroxinemia resulting in low T3 in the fetal brain during the period of neuronal cell migration (weeks 8–12 of pregnancy) may produce morphological brain changes leading to autism.

Insufficient dietary iodine intake and a number of environmental antithyroid and goitrogenic agents can affect maternal thyroid function during pregnancy. The most common causes could include inhibition of deiodinases D2 or D3 from maternal ingestion of dietary flavonoids or from antithyroid environmental contaminants. Some plant isoflavonoids have profound effects on thyroid hormones and on the hypothalamus–pituitary axis. Genistein and daidzein from soy (*Glycine max*) inhibit thyroperoxidase that catalyzes iodination and thyroid hormone biosynthesis. Other plants with hypothyroid effects include pearl millet (*Pennisetum glaucum*) and fonio millet (*Digitaria exilis*); thiocyanate is found in Brassicae plants including cabbage, cauliflower, kale, rutabaga, and kohlrabi, as well as in tropical plants such as cassava, lima beans, linseed, bamboo shoots, and sweet potatoes. Tobacco smoke is also a source of thiocyanate.

Environmental contaminants interfere with thyroid function including 60% of all herbicides, in particular 2,4-dichlorophenoxyacetic acid (2,4-D), acetochlor, aminotriazole, amitrole, bromoxynil, pendamethalin, mancozeb, and thioureas. Other antithyroid agents include polychlorinated biphenyls (PCBs), perchlorates, mercury, and coal derivatives such as resorcinol, phthalates, and anthracenes. A leading ecological study in Texas has correlated higher rates of autism in school districts affected by large environmental releases of mercury from industrial sources. Mercury is a well known antithyroid substance causing inhibition of deiodinases and thyroid peroxidase. The current surge of autism could be related to transient maternal hypothyroxinemia resulting from dietary and/or environmental exposure to antithyroid agents. Additional multidisciplinary epidemiological studies will be required to confirm this environmental hypothesis of autism.

Keywords: Autism; Hypothyroxinemia; Pregnancy; Antithyroid agents; Iodine; Endemic cretinism; Herbicides; Neuronal migration; Soy; Mercury; Polyphenols

1. Introduction

The Centers for Disease Control and Prevention (CDC) estimate at half million the number of individuals with autism and autism spectrum disorders in the United States [1]. From a prevalence of 0.4 to 1/1000 children aged 8 years in the 1980s,

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Mercury Levels in Newborns and Infants After Receipt of Thimerosal-Containing Vaccines

To the Editor.—

Magos et al[1] found that in Porton-Wistar rats the overall brain mercury concentration was lower after exposure to ethyl mercury than to methyl mercury. However, it is significant to note that this study also demonstrated that a higher proportion of inorganic mercury was retained in the brain after exposure to ethyl mercury than to methyl mercury, in each case the inorganic mercury likely being formed as a result of the dealkylation of the ethyl or methyl mercury in the brain. In fact, the absolute concentration of inorganic mercury found in the brain was higher after exposure to ethyl mercury. Furthermore, in addition to the findings commented on by Pichichero et al[2] in the February 2008 *Pediatrics Electronic Pages*, thorough examination of the data from Burbacher et al[3] demonstrates that in infant *Macaca fascicularis* monkeys a higher proportion of mercury from thimerosal-containing vaccines was retained in the brain as inorganic mercury than from oral dosing of methyl mercury; this time, approximately the same level of inorganic mercury was found in both cases.

As previously discussed,[4] once inorganic mercury has found its way into the brain, it has a half-life therein considerably longer than that of ethyl mercury or methyl mercury and has potential to accumulate in cases of repeated or prolonged exposure. Thus, although ethyl mercury and methyl mercury may exhibit more acute neurotoxicity than inorganic mercury, they do not share the same ability to accumulate over time that inorganic mercury exhibits (Vahter et al[5]). Thus, when one considers whether ethyl mercury from thimerosal might play an etiologic role in the development of autism, surely one should also consider that thimerosal may exert toxic effects by contributing to an inorganic mercury load of long half-life in the brain that originates from multiple sources of mercury (including but not limited to elemental mercury from dental amalgam; methyl mercury from fish consumption; elemental mercury and methyl mercury that may have crossed the placenta in utero; methyl mercury in breast milk; and other environmental exposures).

Although the Pichichero et al[2] study provided data regarding the toxicokinetics of ethyl mercury in newborns, it did not provide any insight into the toxicity or toxicodynamics of the small quantities of long half-life inorganic mercury that likely resulted from the dealkylation of ethyl mercury in the central nervous systems of these children. Furthermore, they did not consider that these small quantities of long half-life inorganic mercury may be additive to a preexisting central nervous system load of inorganic mercury of indeterminate amount.

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In Reply.—

Our study was not a toxicology evaluation of ethyl mercury in children; it was a pharmacokinetic study. We are aware of the works cited by Rooney and interpret the findings as he does in the animals evaluated; their applicability to humans is unknown. We certainly could not perform brain biopsies on the normal children we studied to determine if any mercury was present. Furthermore, some mercury in the children's blood was clearly methyl mercury, so even if we performed a brain biopsy it would remain unknown whether the source was methyl or ethyl mercury. We did study the kidneys for evidence of toxicity, because we could obtain urine

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Short Communication

The Endothelium-Dependent Effects of Thimerosal on Mouse Pial Arterioles In Vivo: Evidence for Control of Microvascular Events by EDRF as well as Prostaglandins

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Summary: Thimerosal causes synthesis and/or release of both endothelium-derived relaxing factor (EDRF) and prostaglandins from conductance vessels in vitro. We tested its effects and mechanism of action on mouse pial arterioles in vivo using intravital microscopic techniques. Topical thimerosal dilated pial arterioles. This effect was eliminated by endothelial injury produced by a laser/Evans blue technique. Dilation was also eliminated by topical L-NMMA, a reported inhibitor of EDRF synthesis. Topical thimerosal also reduced the incidence of platelet adhesion/aggregation ("capture") at a site of minimal endothelial damage. This effect was eliminated by L-NMMA pretreatment. The ability of thimerosal to dilate arterioles was eliminated not only by treatments thought to eliminate synthesis/release of EDRF, but also by cyclooxygenase inhibitors. However, inhibition of platelet adhesion/aggregation was not affected by cyclooxygenase inhibition. Thimerosal significantly increased production of prostaglandin E_2 recovered from a closed cranial window. We conclude that the dilating effects of thimerosal on diameter require two endothelium-derived agents: EDRF and one or more prostaglandins acting in concert. However, the inhibiting effect of thimerosal on local platelet adhesion/aggregation appears to be caused only by an increase in EDRF at the injured site. **Key Words:** Thimerosal—EDRF—Prostaglandins—Vasodilation—Brain microcirculation—Endothelial injury—Platelet adhesion/aggregation.

We have published evidence that "classical" endothelium-dependent relaxing factor (EDRF) plays a role both in modulating the tone of mouse pial arterioles in vivo and in modifying the ability of the endothelium of these vessels to attract platelets or initiate platelet aggregation (Rosenblum, 1986, 1988; Rosenblum et al., 1987, 1990*b;* Nishimura et al., 1991). We designate this EDRF as $EDRF_{ACh}$ to signify that it was originally shown to mediate relaxation by acetylcholine (ACh) (Furchgott, 1983).

Thimerosal (sodium ethylmercurithiosalicylate) activates the synthesis and/or release of both "classical" endothelium-dependent relaxing factor and prostaglandins (PG) (Forstermann et al., 1986). In the present studies, we took advantage of these properties of thimerosal to investigate the relative importance of $EDRF_{ACh}$ and PGs in modulating microvascular events in the mouse brain in vivo.

METHODS

Our methods have been described in great detail in numerous publications (Rosenblum and Zweifach, 1963; Rosenblum, 1971; Rosenblum and Nelson, 1988*a,b*; 1990; Rosenblum et al., 1990*b*). In brief, ICR male mice were anesthetized with urethane and their cerebral surface arterioles (pial arterioles) exposed by craniotomy. The mice were maintained at 37°C and the cerebral surface was continuously suffused with mock cerebrospinal fluid (Elliott and Jasper, 1949) at pH 7.3–7.4. All solutions applied to the surface are maintained within this pH range. Television microscopy and an image splitter were used to monitor and measure the selected segment (Baez, 1966).

Selective injury of the endothelium was produced by a

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Abbreviations used: ASA, acetylsalicylic acid; EDRF, endothelium-derived relaxing factor; INDO, indomethacin; L-NMMA, *N*-guanidino-L-monomethyl arginine; PG, prostaglandin.

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Full Length Article

Sex- and structure-specific differences in antioxidant responses to methylmercury during early development

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ABSTRACT

Methylmercury (MeHg) is a ubiquitous environmental contaminant and neurotoxin, particularly hazardous to developing and young individuals. MeHg neurotoxicity during early development has been shown to be sex-dependent via disturbances in redox homeostasis, a key event mediating MeHg neurotoxicity. Therefore, we investigated if MeHg-induced changes in key systems of antioxidant defense are sex-dependent. C57BL/6J mice were exposed to MeHg during the gestational and lactational periods, modeling human prenatal and neonatal exposure routes. Dams were exposed to 5 ppm MeHg via drinking water from early gestational period until postnatal day 21 (PND21). On PND21 a pair of siblings (a female and a male) from multiple (5–6) litters were euthanized and tissue samples were taken for analysis. Cytoplasmic and nuclear extracts were isolated from fresh cerebrum and cerebellum and used to determine thioredoxin (Trx) and glutathione (GSH) levels, as well as thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) activities. The remaining tissue was used for mRNA analysis. MeHg-induced antioxidant response was not uniform for all the analyzed antioxidant molecules, and sexual dimorphism in response to MeHg treatment was evident for TrxR, Trx and GPx. The pattern of response, namely a decrease in males and an increase in females, may impart differential and sex-specific susceptibility to MeHg. GSH levels were unchanged in MeHg treated animals and irrespective of sex. Trx was reduced only in nuclear extracts from male cerebella, exemplifying a structure-specific response. Results from the gene expression analysis suggest posttranscriptional mechanism of sex-specific regulation of the antioxidant response upon MeHg treatment. The study demonstrates for the first time sex-and structure-specific changes in the response of the thioredoxin system to MeHg neurotoxicity and suggests that these differences in antioxidant responses might impart differential susceptibility to developmental MeHg exposure.

1. Introduction

Methylmercury (MeHg) is an environmental pollutant that targets the central nervous system (CNS) and causes severe neurological deficits (Bisen-Hersha et al., 2014; Fischer et al., 2008; Manfroi et al., 2004; Sanfeliu et al., 2003). This is particularly true for newborn and young individuals, which are more susceptible to the toxin due to undeveloped blood-brain barrier (BBB) and lower excretion capacity (Fischer et al., 2008; Manfroi et al., 2004). Targeting the brain by MeHg during early periods of development, when critical processes, such as cell division and neuronal migration take place, leads to irreversible damage, as shown in numerous epidemiological (Llop et al., 2013) and experimental studies (Fischer et al., 2008; Gimenez-Llort et al., 2001; Manfroi et al., 2004). It is noteworthy that sexual dimorphism in response to developmental MeHg exposures has been reported, with males showing increased susceptibility to MeHg than females in behavioral evaluations (Björklund et al., 2007; Gimenez-Llort et al., 2001; Llop et al., 2013; Rossi et al., 1997). However, because of scarce biochemical data, the mechanisms underlying these differences have yet to be elucidated.

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CLINICAL AND LABORATORY OBSERVATIONS

Iatrogenic exposure to mercury after hepatitis B vaccination in preterm infants

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Thimerosal, a derivative of mercury, is used as a preservative in hepatitis B vaccines. We measured total mercury levels before and after the administration of this vaccine in 15 preterm and 5 term infants. Comparison of pre- and post-vaccination mercury levels showed a significant increase in both preterm and term infants after vaccination. Additionally, post-vaccination mercury levels were significantly higher in preterm infants as compared with term infants. Because mercury is known to be a potential neurotoxin to infants, further study of its pharmacodynamics is warranted. (J Pediatr 2000;136;679-81)

The mercury content of drugs and vaccines is being scrutinized, given the potential effects of exposure to mercury through diet and the environment.[1-3] Thimerosal, an organic mercury compound, is used for the enhancement of product stability in several drugs and vaccines. Most neonates received hepatitis B vaccine, which contained thimerosal. The recommended dose of pediatric hepatitis B vaccine contained thimerosal 1:20,000, or 0.25 ppm (12.5 μg of mercury). At our institution the hepatitis B vaccine (at the time of this study) was given within the first week of life, regardless of the mother's hepatitis status. To our knowledge, and according to both manufacturers of the vaccine, no study has examined total mercury levels in newborn infants after inoculation with hepatitis B vaccine. The goal of this study was to evaluate iatrogenic exposure to mercury in preterm infants receiving their initial dose of hepatitis B vaccine in comparison with term infants.

See related articles, p. 571 and p. 599.

METHODS

The study protocol was approved by the Emory Institutional Review Board, and informed written consent was obtained from a parent or guardian for every newborn infant (n = 23) enrolled in the study from Grady Health System's Neonatal Intensive Care Unit between August 1997 and March 1998. All intravenous fluids and medications administered were mercury-free. The inclusion criteria included a birth weight of ≤1000 g, 5-minute Apgar score of 7 or greater, mother who was seronegative for hepatitis B, and hepatitis B vaccine inoculation in the first week of life after consent had been obtained. The control group was composed of 5 term infants whose inclusion criteria differed only by weight of ≥3500 g. Control subjects were not selected from the normal nursery because healthy babies would have been discharged before the post-vaccination levels could be obtained. In the group of 18

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Novel Lipid-Soluble Thiol-Redox Antioxidant and Heavy Metal Chelator, *N,N′*-bis(2-Mercaptoethyl)Isophthalamide (NBMI) and Phospholipase D-Specific Inhibitor, 5-Fluoro-2-Indolyl Des-Chlorohalopemide (FIPI) Attenuate Mercury-Induced Lipid Signaling Leading to Protection Against Cytotoxicity in Aortic Endothelial Cells

Jordan D. Secor, **Sainath R. Kotha**, **Travis O. Gurney**, **Rishi B. Patel**, **Nicholas R. Kefauver**, **Niladri Gupta**, **Andrew J. Morris**, **Boyd E. Haley**, and **Narasimham L. Parinandi**

Abstract

Here, we investigated thiol-redox-mediated phospholipase D (PLD) signaling as a mechanism of mercury cytotoxicity in mouse aortic endothelial cell (MAEC) in vitro model utilizing the novel lipid-soluble thiol-redox antioxidant and heavy metal chelator, *N,N′*-bis(2-mercaptoethyl)isophthalamide (NBMI) and the novel PLD-specific inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI). Our results demonstrated (*i*) mercury in the form of mercury(II) chloride, methylmercury, and thimerosal induced PLD activation in a dose- and time-dependent manner; (*ii*) NBMI and FIPI completely attenuated mercury- and oxidant-induced PLD activation; (*iii*) mercury induced upstream phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2) leading to downstream threonine phosphorylation of PLD_1 which was attenuated by NBMI; (*iv*) mercury caused loss of intracellular glutathione which was restored by NBMI; and (*v*) NBMI and FIPI attenuated mercury- and oxidant-induced cytotoxicity in MAECs. For the first time, this study demonstrated that redox-dependent and PLD-mediated bioactive lipid signaling was involved in mercury-induced vascular EC cytotoxicity which was protected by NBMI and FIPI.

Keywords

mercury; vasculotoxicity; PLD; endothelial cell; NBMI; thiol redox; antioxidant; FIPI; mercaptoethylisophthalamide; bioactive lipid signaling

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Declaration of Conflicting Interests

Boyd E. Haley, a coauthor of this communication, is Professor Emeritus in the Department of Chemistry at the University of Kentucky, Lexington, KY, USA with a 20% research appointment and is on an active NSF grant in that institution. Dr Haley is also the President of CTI Science which is a possible conflict of interest since they hold the license to the patents concerning the compound, NBMI. However, this research project had not been funded or had not been supported or carried out in part or full by CTI Science in any way. Neither Dr Haley nor CTI Science had any influence or control on this research project. This research project was entirely initiated and supervised by Narasimham L. Parinandi at the Ohio State University, Columbus, OH, USA, with the compound, NBMI synthesized by Niladri Gupta (a graduate student of Boyd E. Haley) in the Department of Chemistry at the University of Kentucky, Lexington, KY, USA, under the supervision of Dr Haley. Boyd E. Haley is Chair of the Scientific Advisory Committee of the International Academy of Oral Medicine and Toxicology (IAOMT), who advises the IAOMT Board regarding scientific matters, but he does not have any control over IAOMT in their grant funding decisions. The IAOMT award has been given exclusively to Narasimham L. Parinandi to conduct research without any influence or control of Boyd E. Haley on this research project. Overall, there are no conflicts of interest.

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ANCESTRY OF PINK DISEASE (INFANTILE ACRODYNIA) IDENTIFIED AS A RISK FACTOR FOR AUTISM SPECTRUM DISORDERS

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Pink disease (infantile acrodynia) was especially prevalent in the first half of the 20th century. Primarily attributed to exposure to mercury (Hg) commonly found in teething powders, the condition was developed by approximately 1 in 500 exposed children. The differential risk factor was identified as an idiosyncratic sensitivity to Hg. Autism spectrum disorders (ASD) have also been postulated to be produced by Hg. Analogous to the pink disease experience, Hg exposure is widespread yet only a fraction of exposed children develop an ASD, suggesting sensitivity to Hg may also be present in children with an ASD. The objective of this study was to test the hypothesis that individuals with a known hypersensitivity to Hg (pink disease survivors) may be more likely to have descendants with an ASD. Five hundred and twenty-two participants who had previously been diagnosed with pink disease completed a survey on the health outcomes of their descendants. The prevalence rates of ASD and a variety of other clinical conditions diagnosed in childhood (attention deficit hyperactivity disorder, epilepsy, Fragile X syndrome, and Down syndrome) were compared to well-established general population prevalence rates. The results showed the prevalence rate of ASD among the grandchildren of pink disease survivors (1 in 25) to be significantly higher than the comparable general population prevalence rate (1 in 160). The results support the hypothesis that Hg sensitivity may be a heritable/genetic risk factor for ASD.

Pink disease, or infantile acrodynia as it was also known (primarily in Europe and America), was an especially prevalent condition in Australia, North America, and Central Europe in the first half of the 20th century (Rocaz 1933). The first description of pink disease in the literature dates back to 1903 by Selter, a German physician, although cases in Australia predate this time by at least two decades (Selter 1903; Wood and Wood 1935). Pink disease remained in relative obscurity in the greater medical community until 1914, when it was again described, this time by Swift, an Australian-born physician, at an Australasian medical congress in New Zealand (Swift 1914).

Case studies provided a comprehensive clinical picture of pink disease long before its etiology was established. The most commonly reported symptoms included: irritability, neurosis, photophobia (light sensitivity), hyperhidrosis (excessive sweating), hypotonia (low muscle tone), ataxia (lack of coordination), digestive problems (including loss of weight, loss of appetite, vomiting, and constipation), anemia, excessive salivation, respiratory problems, lethargy, extreme misery, slurring/loss

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Research Article

Thimerosal-Derived Ethylmercury Is a Mitochondrial Toxin in Human Astrocytes: Possible Role of Fenton Chemistry in the Oxidation and Breakage of mtDNA

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Thimerosal generates ethylmercury in aqueous solution and is widely used as preservative. We have investigated the toxicology of Thimerosal in normal human astrocytes, paying particular attention to mitochondrial function and the generation of specific oxidants. We find that ethylmercury not only inhibits mitochondrial respiration leading to a drop in the steady state membrane potential, but also concurrent with these phenomena increases the formation of superoxide, hydrogen peroxide, and Fenton/Haber-Weiss generated hydroxyl radical. These oxidants increase the levels of cellular aldehyde/ketones. Additionally, we find a five-fold increase in the levels of oxidant damaged mitochondrial DNA bases and increases in the levels of mtDNA nicks and blunt-ended breaks. Highly damaged mitochondria are characterized by having very low membrane potentials, increased superoxide/hydrogen peroxide production, and extensively damaged mtDNA and proteins. These mitochondria appear to have undergone a permeability transition, an observation supported by the five-fold increase in Caspase-3 activity observed after Thimerosal treatment.

1. Introduction

1.1. Thimerosal and Ethylmercury. Thimerosal is a preservative that is widely used in medical products, including as a preservative in vaccines, immunoglobulin preparations, skin test antigens, antivenins, ophthalmic and nasal products, and tattoo inks, and is composed of 49.6 percent ethylmercury by weight [1]. The widespread use of Thimerosal exposes many to its potential toxic effects, especially *in utero* and in neonates. We report the results of a series of experiments using cultured normal human astrocytes (NHA) exposed to Thimerosal to study the compound's effect on astrocyte mitochondria.

1.2. Oxidative Stress and Brain. The brain utilizes 20% of the oxygen consumed by the body but constitutes only 2% of the body's mass [2]. Some 5% of molecular oxygen consumption may arise from its reduction to superoxide [3]. The majority of superoxide generated in cells comes from the reaction of molecular oxygen with flavin or quinone radicals, which are partly generated during respiration within complexes of the mitochondrial respiratory chain [4]. The rate of reactive oxygen species (ROS) production increases steeply with increased mitochondrial membrane potential [3]. Superoxide has a very short half-life in cells as it is rapidly dismutased by either the cytosolic Cu-Zn superoxide dismutase (SOD) or the Mn-SOD in the mitochondrial matrix, producing molecular oxygen and hydrogen peroxide. Thus, generation of superoxide is always accompanied by hydrogen peroxide production, and so opens up the possibility of hydroxyl radical ($HO^{\bullet}$) generation via Fenton/Haber-Weiss chemistry [5]. Fenton metals, including iron and copper, catalyze the production of $HO^{\bullet}$ from superoxide/hydrogen peroxide and so the free, unchelated levels of transition metals inside cells are very low and normally all stored in an oxidized

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Research Article

B-Lymphocytes from a Population of Children with Autism Spectrum Disorder and Their Unaffected Siblings Exhibit Hypersensitivity to Thimerosal

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The role of thimerosal containing vaccines in the development of autism spectrum disorder (ASD) has been an area of intense debate, as has the presence of mercury dental amalgams and fish ingestion by pregnant mothers. We studied the effects of thimerosal on cell proliferation and mitochondrial function from B-lymphocytes taken from individuals with autism, their nonautistic twins, and their nontwin siblings. Eleven families were examined and compared to matched controls. B-cells were grown with increasing levels of thimerosal, and various assays (LDH, XTT, DCFH, etc.) were performed to examine the effects on cellular proliferation and mitochondrial function. A subpopulation of eight individuals (4 ASD, 2 twins, and 2 siblings) from four of the families showed thimerosal hypersensitivity, whereas none of the control individuals displayed this response. The thimerosal concentration required to inhibit cell proliferation in these individuals was only 40% of controls. Cells hypersensitive to thimerosal also had higher levels of oxidative stress markers, protein carbonyls, and oxidant generation. This suggests certain individuals with a mild mitochondrial defect may be highly susceptible to mitochondrial specific toxins like the vaccine preservative thimerosal.

1. Introduction

Autism spectrum disorder (ASD) is a complex developmental disorder characterized by abnormalities of verbal and nonverbal communication, stereotyped restricted interests, repetitive behavioral patterns, and impairment of socialization. ASD now affects 1 in 88 children in the USA [1, 2]. In Great Britain, the costs of supporting children with ASD amount to be £2.7 bil/yr, while for adults these costs amount to £25 bil/year [3]. Recent studies have estimated that the lifetime cost to care for an individual with an ASD is $3.2 mil [4]. In the USA individuals with ASD have medical expenditures 4.1–6.2x greater than those without ASD, with median expenditures being almost 9 times greater [5, 6]. ASD is usually diagnosed before 4 years of age and has a 5 : 1 male to female gender bias. Although it is believed that multiple interacting genetic and environmental factors influence individual vulnerability to ASD, none have been reproducibly identified in more than a fraction of cases. In addition to complex gene-environment interactions, the heterogeneous presentation of behavioral symptoms within the spectrum of autistic disorders suggests a variable and multifactorial pathogenesis.

Mercury. Mercury is a ubiquitous environmental contaminant, that is, transformed into the volatile neurotoxins methylmercury and ethylmercury. In the United States, more than 8500 water bodies in 45 states and territories are listed as impaired for Hg in water, sediments, and/or fish tissue, including many sites lacking a point source of Hg pollution [7]. In addition to the environmental inorganic/organic mercury assaults many children have been exposed to ethylmercury in the form of thimerosal (called thiomersal in the UK, marketed as Merthiolate in the USA) has been used as a preservative agent for vaccines and toxoids [8]. The relationship between thimerosal and ASD has become a very debated topic over the last decade and some researchers have

MERCURY, VACCINES, AND AUTISM, REVISITED

Baker's recent article[1] presented fascinating insights and perspectives on the intertwined stories of mercury, vaccines, and autism. As a researcher in mercury with some involvement in the autism issue (as a participant in expert committees for the National Institutes of Health, the National Research Council, and the Environmental Protection Agency [EPA] as well as an invited reviewer of a proposed clinical trial of mercury chelation in autism), I would like to offer some additions to an excellent article.

First, it is not entirely correct to suggest that there is no medical knowledge of the potential hazards associated with thimerosal apart from a convergence with the history of knowledge about methyl mercury. Baker omits the separate (but convergent) history of toxicities associated with thimerosal in topical medicines, such as contact lens solution, eye drops, and other products. The literature on this history (first reviewed in 1981[2]) prompted restrictions on the use of thimerosal in these products by the Food and Drug Administration (FDA) in 1998.

Second, most methyl mercury exposure occurs because of the consumption of fish that has been exposed to environmental biomethylation. Methyl mercury has not been used in paints or pesticides; the organomercurials that have been used in these products are methoxy ethyl mercury chloride and phenyl mercury compounds.[3]

Third, although there is experimental evidence that ethylmercury behaves differently from methyl mercury in terms of toxicokinetics,[4] it appears to have qualitatively similar effects on the nervous and immune systems.[5,6]

Finally, an additional force for convergence not mentioned by Baker is the coincidence of the increasing number of early childhood vaccinations with the increasing knowledge of low-level organomercury toxicity.

Although Baker's article is informative, I read history somewhat differently. I believe it illustrates an additional lesson not noted in the article: much trouble could have been avoided if the FDA had made a prudent decision early in the controversy to reduce infant exposures to mercury compounds by removing thimerosal from medications. Such a decision should have been made at least by 1997, when the EPA issued its report to Congress on mercury.[7]

This path would have emulated the voluntary cessation of lead soldering in food cans, which the FDA encouraged and the food industry undertook in the early 1970s without a prolonged debate on whether this specific use was associated with specific neurotoxic outcomes in children. To quote my former mentor J. Julian Chisolm, "one should not shy from introducing interim measures" even if they are partial.[8] Mercury, like lead, is a chronic and accumulative toxin, and reductions in any source have a public health benefit. No discussion of specific associations with autism would have been necessary, and much heartache could have been avoided. The increased suspicion of the public toward the biomedical profession, the drug industry, and the regulatory community could have been avoided as well.

Moreover, the intensity of the advocacy response, particularly by parents of children with autism, should be seen in the context of the lack of attention to preventable risk factors for autism, which is clearly not a genetically determined disease although genetic susceptibility may play an important role in modulating response to acquired risks In this sense, mercury may be seen as symbolic of the importance of environmental risk factors (defined broadly and not just chemically) as well as of the lack of a research agenda at the national level for autism despite its status as a major neurodevelopmental disorder of children. ■

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Mercury as a possible link between maternal obesity and autism spectrum disorder

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ABSTRACT

The incidence of both obesity and autism spectrum disorders (ASD) has dramatically increased during the last decades. Moreover, the most recent studies have revealed increased risk of ASD in offspring of overweight and obese women. However, the mechanisms of association between ASD and maternal obesity are unknown. Taking into account the existing data indicating the association between mercury (Hg) exposure and development of obesity and ASD, we hypothesize that Hg may serve as an additional link between maternal obesity and ASD. In particular, it is supposed that obesity is associated with excessive accumulation of Hg in the maternal organism. After conception, the fetus is developing in the conditions of Hg overload within the body of obese women thus predisposing to the development of ASD. The proposed hypothesis may be confirmed by the existing data. In particular, previous studies demonstrated that overweight and obese persons are characterized by a significantly higher level of Hg in hair, blood and urine than the lean ones. Therefore, an obese organism is characterized by elevated Hg burden that may be transferred to the fetus during pregnancy. Moreover, multiple studies have demonstrated a tight association between maternal and children Hg status being indicative of placental transfer of metal from maternal organism to offspring. Finally, a growing body of data indicates the influence of Hg exposure and Hg status on the risk of ASD in children. However, additional experimental and clinical studies are required to prove the hypothesis and provide novel data on the role of Hg in maternal obesity-associated ASD development. In particular, the contribution of Hg to ASD development in children from obese mothers should be determined. If a significant role of Hg in maternal obesity ASD risk will be confirmed, this will open additional perspectives of risk modification. Taking into account the universal mechanisms of Hg toxicity, transport, and accumulation, further preventive actions may be undertaken to reduce the risk of Hg toxicity and Hg-associated ASD development. In particular, it is supposed that the use of Hg chelators (like N,N′bis-(2-mercaptoethyl)isophthalamide, NMBI), antioxidants, and anti-inflammatory compounds prior or during pregnancy may have a beneficial effect. However, the safety of such actions should repeatedly be tested to avoid adverse health effects in a developing fetus.

Hypothesis

We hypothesize that Hg may serve as an additional link between maternal obesity and ASD. In particular, it is supposed that obesity is associated with excessive accumulation of Hg in the maternal organism. After conception, the fetus is developing in the conditions of Hg overload within the body of obese women thus predisposing to the development of ASD.

Review of evidential support

Obesity epidemiology

Obesity is a metabolic disorder considered to be a worldwide epidemic [1]. It has reached epidemic proportions in developed

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Research report

Inhibitory action of thimerosal, a sulfhydryl oxidant, on sodium channels in rat sensory neurons

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Abstract

The effects of thimerosal, a sulfhydryl oxidizing agent, on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium channels in rat dorsal root ganglion neurons were studied using the whole-cell patch clamp technique. Thimerosal blocked the two types of sodium channels in a dose-dependent manner. The inhibitory effect of thimerosal was much more pronounced in TTX-R sodium channels than TTX-S sodium channels. The effect of thimerosal was irreversible upon wash-out with thimerosal-free external solution. However, dithiothreitol, a reducing agent, partially reversed it. Thimerosal shifted the steady-state inactivation curves for both types of sodium channels in the hyperpolarizing direction. The voltage dependence of activation of both types of sodium channels was shifted in the depolarizing direction by thimerosal. The inactivation rate in both types of sodium channels increased after thimerosal treatment. All these effects of thimerosal would add up to cause a depression of sodium channel function leading to a diminished neuronal excitability.

Themes: Excitable membranes and synaptic transmission

Topics: Sodium channels

Keywords: Sulfhydryl oxidation; Thimerosal; Tetrodotoxin-sensitive; Tetrodotoxin-resistant; Sodium channel; Dorsal root ganglion

1. Introduction

Voltage-gated sodium channel plays an important role in generation and conduction of action potential in excitable cells. Sodium channels on the axon initial segment of neurons determine the threshold for the action potential and affect the duration and frequency of repetitive firings. Also the release of neurotransmitters from presynaptic nerve terminal is influenced by sodium channel activity. The function of sodium channels is subject to modulation by various toxins, therapeutic drugs and neuromodulators.

Tetrodotoxin (TTX) is a potent neurotoxin that blocks voltage-gated sodium channels. Most sodium channels are blocked by TTX at the concentration range of 1–10 nM. However, sodium channels that are not sensitive to TTX exist in various tissues and in different animal species [32]. Rat dorsal root ganglion (DRG) neurons are endowed with TTX-sensitive (TTX-S) as well as TTX-resistant (TTX-R) sodium channels [9,15,17]. Compared to TTX-S sodium current TTX-R sodium current exhibits slower time course of activation and inactivation, activates at higher voltage, and has a smaller single channel conductance. Pharmacologically TTX-R sodium channels are more sensitive to divalent cations (Co^{2+}, Mn^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}) and pyrethroid insecticide but less sensitive to lidocaine than TTX-S sodium channels [20,21,25,28]. The TTX-R sodium channel in DRG neurons was cloned and its amino acid sequence revealed some homology with a cardiac sodium channel. According to in situ hybridization this channel was localized to DRG cells with smaller diameters [2,22,23].

Protein cysteine residues are reactive to the cellular redox state and participate in the regulation of cellular functions. The redox modification of cysteine sulfhydryl groups has been shown to alter the function of various ion channels. The activity of voltage-dependent potassium channels was increased by oxidation but decreased by

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Screening Identifies Thimerosal as a Selective Inhibitor of Endoplasmic Reticulum Aminopeptidase 1

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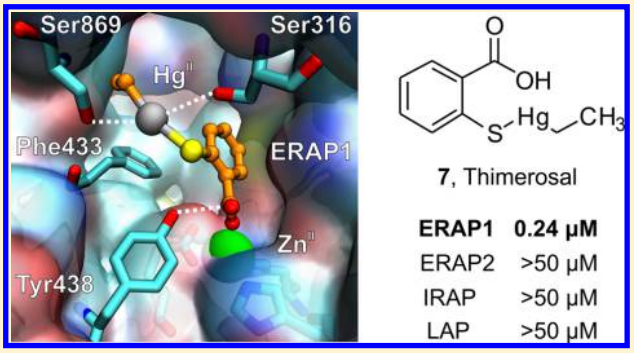
Supporting Information

ABSTRACT: We employed virtual screening followed by *in vitro* evaluation to discover novel inhibitors of ER aminopeptidase 1, an important enzyme for the human adaptive immune response that has emerged as an attractive target for cancer immunotherapy and the control of autoimmunity. Screening hits included three structurally related compounds carrying the (*E*)-*N*′-((1*H*-indol-3-yl)methylene)-1*H*-pyrazole-5-carbohydrazide scaffold and (2-carboxylatophenyl)sulfanyl-ethylmercury as novel ERAP1 inhibitors. The latter, also known as thimerosal, a common component in vaccines, was found to inhibit ERAP1 in the submicromolar range and to present strong selectivity versus the homologous aminopeptidases ERAP2 and IRAP. Cell-based analysis indicated that thimerosal can effectively reduce ERAP1-dependent cross-presentation by dendritic cells in a dose-dependent manner.



KEYWORDS: *ERAP1, ERAP2, IRAP, aminopeptidase, inhibitor, immune system, antigenic peptide, docking*

Endoplasmic reticulum (ER) aminopeptidases generate antigenic peptides for loading onto Major Histocompatibility Class I molecules (MHCI), which then interact with receptors on cytotoxic T-lymphocytes to initiate adaptive immune responses against infected or cancerous cells.[1,2] ER aminopeptidase 1 (ERAP1) is particularly effective in this function, and many *in vitro* and *in vivo* studies have established its role in regulating adaptive immune responses. For these reasons, ERAP1 is an attractive target for both cancer immunotherapy and the control of autoimmune reactions.[3,4] Indeed, ERAP1 down-regulation by available inhibitors has been reported to enhance cytotoxic responses versus cancer and suppress cellular autoimmune responses in Ankylosing Spondylitis.[4−6] Despite its biological importance, however, no clinical application of ERAP1 inhibitors have been reported, in part due to the lack of pharmacologically appropriate potent and selective inhibitors. Bestatin (ubenimex), a typical aminopeptidase inhibitor, has been evaluated in clinical settings but is a poor inhibitor of ERAP1.[7] Recent rational design efforts have yielded promising leads including a phosphinic pseudopeptide nanomolar inhibitor (DG013A, Chart 1) that displayed however low selectivity toward homologous enzymes, and 3,4-diaminobenzoic acid derivatives (such as **3**, Chart 1) that displayed a better selectivity profile albeit with modest potency.[8,9] In an effort to discover novel, nonpeptidic scaffolds that inhibit ERAP1 as leads for preclinical development we applied a combination of structure-based, ligand-based, and knowledge-based virtual screening approaches, taking advantage of key structural characteristics revealed in the recent crystal structures of ERAP1 and ERAP2 and their complexes with **1** and **2**, respectively.[8,10,11]

Toward this goal, we compiled a library of more than 265,000 compounds from selected collections of chemical vendors that are focused on drug-likeness and structural diversity (Table S1). The library was enriched with the National Cancer Institute's diversity set II (1364 compounds) and the DrugBank database comprising 6590 FDA-approved and experimental small-molecule drugs[12] We also performed a 3D pharmacophore search against the purchasable subset of the ZINC database (more than 20 million compounds)[13] using the online interface of ZINCPharmer.[14] The pharmacophore features of the query were extracted from the X-ray crystal structures of ERAP1 complex with bestatin and ERAP2 complex with DG013A,[8,10,11] which were further refined to a consensus pharmacophore (see the Computational Methods section, Table S2, and Figure S1 in the Supporting Information for more details). The filtered query results (3959 compounds) supplemented our small-molecule library for docking to ERAP1.

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ORIGINAL PAPER

Maternal Thimerosal Exposure Results in Aberrant Cerebellar Oxidative Stress, Thyroid Hormone Metabolism, and Motor Behavior in Rat Pups; Sex- and Strain-Dependent Effects

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Published online: 21 October 2011

Abstract Methylmercury (Met-Hg) and ethylmercury (Et-Hg) are powerful toxicants with a range of harmful neurological effects in humans and animals. While Met-Hg is a recognized trigger of oxidative stress and an endocrine disruptor impacting neurodevelopment, the developmental neurotoxicity of Et-Hg, a metabolite of thimerosal (TM), has not been explored. We hypothesized that TM exposure during the perinatal period impairs central nervous system development, and specifically the cerebellum, by the mechanism involving oxidative stress. To test this, spontaneously hypertensive rats (SHR) or Sprague–Dawley (SD) rat dams were exposed to TM (200 μg/kg body weight) during pregnancy (G10–G15) and lactation (P5–P10). Male and female neonates were evaluated for auditory and motor function; cerebella were analyzed for oxidative stress and thyroid metabolism. TM exposure resulted in a delayed startle response in SD neonates and decreased motor learning in SHR male (22.6%), in SD male (29.8%), and in SD female (55.0%) neonates. TM exposure also resulted in a significant increase in cerebellar levels of the oxidative stress marker 3-nitrotyrosine in SHR female (35.1%) and SD male (14.0%) neonates. The activity of cerebellar type 2 deiodinase, responsible for local intrabrain conversion of thyroxine to the active hormone, 3′,3,5-triiodothyronine (T3), was significantly decreased in TM-exposed SHR male (60.9%) pups. This coincided with an increased (47.0%) expression of a gene negatively regulated by T3, Odf4 suggesting local intracerebellar T3 deficiency. Our data thus demonstrate a negative neurodevelopmental impact of perinatal TM exposure which appears to be both strain- and sex-dependent.

Keywords Ethylmercury · Rat · Cerebellum · Oxidative stress marker 3-nitrotrosine (3-NT) · Type 2 deiodinase (D2)

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Introduction

Environmental toxicants such as heavy metals [1] including mercury Hg [2, 3] have been identified as factors exerting a range of harmful neurological and cognitive effects in humans and experimental animals, and have been implicated in the etiology of a number of neuropsychiatric disorders. The major environmental organic compounds of mercury include methylmercury (Met-Hg) and ethylmercury (Et-Hg). The main exposure to Met-Hg comes from contaminated fish through bioaccumulation of both organic and inorganic of Hg environmental contamination.

Met-Hg accumulates in both fetal and neonatal brains potentially affecting neurodevelopment [4]. Met-Hg has been shown to cross the placenta [5] and can be transferred from plasma to mothers' milk [6]. It is a known trigger of oxidative stress [7, 8] and both an endocrine [9, 10] and antioxidant defense system [11, 12] disruptor. Gestational exposure to Met-Hg in mice results in increased lipid peroxidation and reduced developmental increase in GSH in the brain [13].

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Effects of Thimerosal on Lipid Bilayers and Human Erythrocytes: An In Vitro Study

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Abstract Thimerosal (THI, ethyl-mercury thiosalicylate) is added to vaccines as a preservative; as a consequence, infants may have been exposed to bolus doses of Hg that collectively added up to nominally 200 μg Hg during the first 6 months of life. While several studies report an association between THI-containing vaccines and neurological disorders, other studies do not support the causal relation between THI and autism. With the purpose to understand the molecular mechanisms of the toxic effect of THI it was assayed on human red cells and in bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), classes of phospholipids found in the outer and inner monolayers of the human erythrocyte membrane, respectively. The capacity of THI to interact with DMPC and DMPE was determined by X-ray diffraction and differential scanning calorimetry, whereas intact human erythrocytes were observed by optical, defocusing and scanning electron microscopy. The experimental findings of this study demonstrated that THI interacted in a concentration-dependent manner with DMPC and DMPE bilayers, and in vitro interacted with erythrocytes inducing morphological changes. However, concentrations were considerable higher than those present in vaccines.

Keywords Thimerosal · Human erythrocyte membrane · Lipid bilayer

Abbreviations

THI	Thimerosal
DMPC	Dimirystoylphosphatidylcholine
DMPE	Dimyristoylphosphatidylethanolamine
SEM	Scanning electron microscopy
DM	Defocusing microscopy
DSC	Scanning differential calorimetry

Introduction

Thimerosal (THI, sodium ethyl-mercury thiosalicylate, $C_9H_9HgNaO_2S$, Fig. 1) is 49.55 % Hg in weight. It is currently used in pharmaceutical preparations and as a bactericidal and fungicidal additive to drugs that are injected (Geier et al. 2015). In water solutions, THI dissociates into thiosalicylic acid and ethylmercury cation (Trümpler et al. 2014). Its antimicrobial activity is due to the small amounts of ethylmercury. It has been reported that the in vitro toxicity of ethylmercury and THI is comparable to the toxicity of methylmercury (Dórea et al. 2013). Thimerosal is also used in vaccines as a preservative, at concentrations of 12.5–25 μg Hg per 0.5 mL vaccine dose. As a result, infants can be exposed to bolus doses of Hg in the range of 12.5–62.5 μg Hg that would amount up to 200 μg Hg during the first 6 months of life (Geier et al. 2013). Massive overdoses of products containing THI have resulted in toxic effects (Pichichero et al. 2002). Studies in humans have concluded that THI is

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PLOS ONE

RESEARCH ARTICLE

Methylmercury Causes Blood-Brain Barrier Damage in Rats via Upregulation of Vascular Endothelial Growth Factor Expression

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Abstract

Clinical manifestations of methylmercury (MeHg) intoxication include cerebellar ataxia, concentric constriction of visual fields, and sensory and auditory disturbances. The symptoms depend on the site of MeHg damage, such as the cerebellum and occipital lobes. However, the underlying mechanism of MeHg-induced tissue vulnerability remains to be elucidated. In the present study, we used a rat model of subacute MeHg intoxication to investigate possible MeHg-induced blood-brain barrier (BBB) damage. The model was established by exposing the rats to 20-ppm MeHg for up to 4 weeks; the rats exhibited severe cerebellar pathological changes, although there were no significant differences in mercury content among the different brain regions. BBB damage in the cerebellum after MeHg exposure was confirmed based on extravasation of endogenous immunoglobulin G (IgG) and decreased expression of rat endothelial cell antigen-1. Furthermore, expression of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, increased markedly in the cerebellum and mildly in the occipital lobe following MeHg exposure. VEGF expression was detected mainly in astrocytes of the BBB. Intravenous administration of anti-VEGF neutralizing antibody mildly reduced the rate of hind-limb crossing signs observed in MeHg-exposed rats. In conclusion, we demonstrated for the first time that MeHg induces BBB damage via upregulation of VEGF expression at the BBB *in vivo*. Further studies are required in order to determine whether treatment targeted at VEGF can ameliorate MeHg-induced toxicity.

Introduction

Methylmercury (MeHg) is a by-product formed during acetaldehyde synthesis. MeHg also occurs in nature due to the microbial methylation of mercury. Artificially produced MeHg has caused serious environmental problems over the past 60 years in Japan [1],[2]. Although extensive artificial MeHg pollution has been reduced, the naturally occurring environmental form is increasing due to increasing mercury emission into the atmosphere associated with

Systemic reactions due to thiomersal

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Key words: Thiomersal – vaccine – systemic reaction.

Although thiomersal is a widely used preservative in vaccines, immunoglobulins and intracutaneous test solutions, only 1 case of systemic reaction to this compound has been reported (1). We have seen 3 patients presenting a systemic reaction probably due to this preservative.

Case Reports

Case 1

A 66-year-old woman was admitted with acute urticaria 3 days after she had been vaccinated against influenza with a vaccine containing 0.01% thiomersal (INFLUVAC-Duphar). The rash disappeared within a few days without treatment.

Case 2

A 31-year-old doctor developed acute urticaria one day after vaccination against hepatitis B with a vaccine containing 0.05% thiomersal (H-B-VAX- Merk S.D.).

Case 3

A 16-year-old girl had a generalized exanthematic eruption. The history revealed that 2 days before she had received tetanus immunoglobulins containing 0.1% thiomersal (TETUMAN-Berna) following a trivial domestic injury.

Patch tests with ICDRG series (Hermal Trolab) were negative; a patch test with thiomersal 0.1% pet. was positive in all 3 patients after 48 and 72 h.

Discussion

These 3 patients presented a systemic cutaneous reaction after injection of parenteral solutions containing thiomersal as preservative. Since the patch tests revealed sensitization, we believe that thiomersal was the cause. Systemic reactions to thiomersal may not be rare. Some systemic reactions may have been misdiagnosed as being due to the pharmaceutical principles of the drugs. The presence of thiomersal in a large number of a widely used solutions means that exposure to it is extremely common, and sensitization could be frequent (2–4).

It is useful to patch test with thiomersal in all patients with systemic reactions following the use of solutions containing merthiolate.

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Applied methodology

In vitro study of thimerosal reactions in human whole blood and plasma surrogate samples

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ABSTRACT

Because of its bactericidal and fungicidal properties, thimerosal is used as a preservative in drugs and vaccines and is thus deliberately injected into the human body. In aqueous environment, it decomposes into thiosalicylic acid and the ethylmercury cation. This organomercury fragment is a potent neurotoxin and is suspected to have similar toxicity and bioavailability like the methylmercury cation. In this work human whole blood and physiological simulation solutions were incubated with thimerosal to investigate its behaviour and binding partners in the blood stream. Inductively coupled plasma with optical emission spectrometry (ICP-OES) was used for total mercury determination in different blood fractions, while liquid chromatography (LC) coupled to electrospray ionisation time-of-flight (ESI-TOF) and inductively coupled plasma-mass spectrometry (ICP-MS) provided information on the individual mercury species in plasma surrogate samples. Analogous behaviour of methylmercury and ethylmercury species in human blood was shown and an ethylmercury-glutathione adduct was identified.

1. Introduction

In 1931, ethylmercury thiosalicylate (thimerosal, THI) was introduced into the market as a bactericidal and fungicidal additive to drugs that are distributed in multi-dose ampullae. Since its market introduction for many decades this mercury-containing agent has been used with little or no attention to possible dangerous effects which might occur upon its intramuscular or intravenous injection into the human body. Although the methylmercury cation ("methylmercury", MeHg) is a strong toxin that is to be avoided even at small levels when consumed in foods such as seafood and rice (in Asia), the World Health Organization considers small doses of thimerosal safe regardless of multiple/repetitive exposures to vaccines that are predominantly taken during pregnancy or infancy. Anyhow, an ongoing discussion about suspected neurotoxic effects [1–3] in patients treated with THI-preserved drugs lead to the recommendation of government organizations towards the pharmaceutical industry to phase out thimerosal as an adjuvant in vaccines in 2001. Temporarily withdrawn from routine childhood vaccination schedules in Europe and the US, thimerosal is still in use in the United States of America and in developing countries, and was present in most anti-flu vaccines against the H1N1 virus (swine influenza) in 2009. In aqueous media, THI undergoes a hydrolysis equilibrium reaction, dissolving into thiosalicylic acid (TSA) and the ethylmercury cation ("ethylmercury", EtHg) [4,5]. Recently Dorea et al. reviewed the toxicity of thimerosal and ethylmercury in comparison to methylmercury [6] and concluded that the *in vitro* toxicity of ethylmercury and thimerosal is comparable with the toxicity of methylmercury, but the different pharmacokinetics leading to a shorter residence time of ethylmercury in the blood warrants special attention for studying the *in vivo* toxicity. However, since the target organ for the toxicity of organic mercury compounds is the brain, the shorter residence time in blood is not necessarily a risk reducing factor.

Due to the strong affinity of mercury towards sulphur and the almost universal presence of this chalcogen in the human body in the form of thiols and disulphides in peptides, proteins and DNA, sulphur is the major binding partner of mercury compounds under physiological conditions. Mercury unfolds its neurotoxic effects by binding to thiols or disulphides in the nervous system thus inhibiting enzyme activities, distorting protein structure or

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Original Paper

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Thimerosal Induces Toxic Reaction in Non-Sensitized Animals

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Abstract

The effects of injection of thimerosal solution on nonsensitized animals was investigated. Intrafootpad injection of thimerosal solution in nonsensitized mice resulted in a swelling response which peaked 1 h after injection and lasted for more than 24 h. Histopathological examination showed that there were severe edema and infiltration of polymorphonuclear neutrophils at the site of injection. An increased vascular permeability was observed after cutaneous injection of thimerosal solution on the back of nonsensitized rats. Since mercuric chloride and methyl mercury induced severer reactions, and thiosalicylic acid had no effect, mercury contained in thimerosal would have caused the reactions observed in this study. These results suggest that part of these hypersensitivity reactions against thimerosal observed among patients were possibly induced by the toxic effect of thimerosal. Therefore, thimerosal contained as a preservative in vaccine may augment the side-effects of the vaccination.

Key Words

Footpad swelling
Histamine release
Mast cells
Thimerosal, vascular pemeability

Introduction

Thimerosal (merthiolate, mercurothiolate), a compound of organic mercury and thiosalicylic acid, has been widely used as a preservative in vaccine, eye drops, and contact lens care solutions. Recently, many investigators [1–4] have reported that thimerosal participates in the allergic reactions such as local swelling, and that immediate-type reactions occur after vaccination. Most of these authors have postulated that thimerosal might act as an allergen after repeated sensitization. A variety of immunopathological effects by mercury compounds are also reported, such as autoantibody formation [5], allergic contact dermatitis [6], alterations of serum immunoglobulin concentrations [7], and autoimmune glomerulonephritis [8], in both animal models [5–7] and in man [8].

In our study, thimerosal elicited an immediate reaction at the site of injection in nonsensitized animals. The reaction is commonly observed after injection of thimerosal and two other mercury compounds, mercuric chloride and methyl mercury, in naive mice and rats with different genetic background. In this report, the toxic reactions observed in nonsensitized animals after injection of thimerosal are demonstrated and the possible mechanism of the induction of toxic reactions after injection with thimerosal is discussed.

Materials and Methods

Animals

Female BALB/c CrSlc, C57BL/6 CrSlc, and C3H/He Slc mice (6 weeks of age) and female Sprague-Dawley (Slc:SD) rats (8 weeks of age) wer purchased from the Shizuoka Laboratory Animals Center, Shizuoka, Japan.

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Effect of thimerosal, a preservative in vaccines, on intracellular Ca^{2+} concentration of rat cerebellar neurons

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Abstract

The effect of thimerosal, an organomercurial preservative in vaccines, on cerebellar neurons dissociated from 2-week-old rats was compared with those of methylmercury using a flow cytometer with appropriate fluorescent dyes. Thimerosal and methylmercury at concentrations ranging from 0.3 to 10 μM increased the intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$) in a concentration-dependent manner. The potency of 10 μM thimerosal to increase the $[Ca^{2+}]i$ was less than that of 10 μM methylmercury. Their effects on the $[Ca^{2+}]i$ were greatly attenuated, but not completely suppressed, under external Ca^{2+}-free condition, suggesting a possibility that both agents increase membrane Ca^{2+} permeability and release Ca^{2+} from intracellular calcium stores. The effect of 10 μM thimerosal was not affected by simultaneous application of 30 μM L-cysteine whereas that of 10 μM methylmercury was significantly suppressed. The potency of thimerosal was similar to that of methylmercury in the presence of L-cysteine. Both agents at 1 μM or more similarly decreased the cellular content of glutathione in a concentration-dependent manner, suggesting an increase in oxidative stress. Results indicate that thimerosal exerts some cytotoxic actions on cerebellar granule neurons dissociated from 2-week-old rats and its potency is almost similar to that of methylmercury.

Keywords: Thimerosal; Vaccine; Preservative; Cerebellar neurons; Calcium

1. Introduction

Thimerosal is an organomercurial preservative in vaccines to prevent contamination with harmful microbes and its derivative is ethylmercury. There is a concern on the part of public health community that adverse health consequences by thimerosal may occur among infants during immunization schedule although it is generally believed that the safety of thimerosal use for humans have been established (Mahaffey, 1999; Ball et al., 2001).

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Toxicology in Vitro

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Property of thimerosal-induced decrease in cellular content of glutathione in rat thymocytes: a flow cytometric study with 5-chloromethylfluorescein diacetate

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Abstract

There is a concern on the part of public health community that adverse health consequences by thimerosal, a preservative in vaccines for infants, may occur among infants during immunization schedule. Therefore, the effect of thimerosal on cellular content of glutathione was examined on thymocytes obtained from 4-week-old rats using a flow cytometer and 5-chloromethylfluorescein diacetate. Thimerosal at concentrations ranging from 1 to 10 μM reduced the cellular content of glutathione in a concentration-dependent manner, and the complete depletion of cellular glutathione was observed when the cells were treated with 30 μM thimerosal. L-Cysteine significantly attenuated the actions of thimerosal to reduce the glutathione content and to increase the intracellular Ca^{2+} concentration. Prolonged incubation (24 h) with 1–3 μM thimerosal induced the apoptosis. The cytotoxic action of thimerosal was greatly augmented when the cells suffered oxidative stress induced by H_2O_2. It may be unlikely that thimerosal exerts potent cytotoxic action under the in vivo condition because the blood concentration of thimerosal after receiving vaccines does not seem to reach micromolar range and nonprotein thiols at micromolar concentrations are present in the blood.

Keywords: Thimerosal; Glutathione; Oxidative stress; Lymphocyte

1. Introduction

Thimerosal has been used as an organomercurial preservative in vaccines to prevent contamination with harmful microbes since early 1930s. There is a recent concern on the part of public health community that adverse health consequences by thimerosal may occur among infants during immunization schedule (Ball et al., 2001; van't Veen, 2001; Westphal and Hallier, 2002; Westphal et al., 2003). In laboratory in vitro studies, thimerosal has been used as a sulfhydryl reagent to modify some of membrane and cellular functions (Bootman et al., 1992; Cai and Sauve, 1997; Marengo et al., 1998; Lang et al., 2000). The action of thimerosal to decrease content of cellular glutathione is probably one of basic actions related to its toxicity because of a following reason. Change in cellular redox status modulates channel and receptor activities in several types of cells (Elliott and Koliwad, 1997; Lipton et al., 1998; Tanaka et al., 1999; Choi and Lipton, 2000; Pessah, 2001). Furthermore, the cell growth and death are related to cellular redox state (Powis et al., 1995; Buttke and Sandstrom, 1995; Hampton and Orrenius, 1998; Mates et al., 2002). Therefore, to elucidate the property of thimerosal-induced action on cellular content of glutathione, we have examined the effect of thimerosal on lymphocytes obtained from rat thymic glands using a flow cytometer with fluorescent dyes.

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Effect of the ophthalmic preservative thimerosal on rabbit and human corneal endothelium

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Widespread use of the mercurial-containing preservative thimerosal as an antibacterial agent in ophthalmic drugs and solutions warranted an investigation into its possible cytotoxic effects on the functional and ultrastructural integrity of the corneal endothelium. No changes in corneal thickness were observed during 5 hours' perfusion of the endothelium of rabbit and human corneas with 0.0001 and 0.0005 percent thimerosal in glutathione bicarbonate Ringer's solution (GBR). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of the endothelium of the 0.0001 percent group revealed normal ultrastructure. SEM and TEM of the endothelium of corneas perfused with 0.0005 percent thimerosal for 5 hours revealed condensed mitochondria, cytoplasmic vacuoles, and cytoplasmic flaps at the apical end of the cellular junctions. Perfusion of higher concentrations (0.001 and 0.005 percent) of thimerosal in GBR resulted in increases in corneal thickness after 2 hours and irreversible ultrastructural damage to the endothelial cells by 5 hours. Corneas perfused with 0.01 and 0.1 percent thimerosal in GBR showed a rapid and immediate increase in corneal thickness and endothelial cell death and necrosis within 1 hour. It is postulated that the mercury in thimerosal becomes bound to the cell membrane protein sulfhydryl groups, causing an increase in cellular permeability. These results suggest that the prolonged exposure of the corneal endothelium to thimerosal in the accepted antimicrobial dosage of 0.005 to 0.001 percent may result in functional and structural damage to the endothelium.

Key words: cornea, corneal endothelium, preservatives, scanning and transmission electron microscopy, thimerosal, toxicity.

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LEADING ARTICLE

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Vaccines Without Thiomersal

Why So Necessary, Why So Long Coming?

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Abstract

The inorganic mercurial thiomersal (merthiolate) has been used as an effective preservative in numerous medical and non-medical products since the early 1930s. Both the potential toxicity of thiomersal and sensitisation to thiomersal in relation to the application of thiomersal-containing vaccines and immunoglobulins, especially in children, have been debated in the literature.

The very low thiomersal concentrations in pharmacological and biological products are relatively non-toxic, but probably not *in utero* and during the first 6 months of life. The developing brain of the fetus is most susceptible to thiomersal and, therefore, women of childbearing age, in particular, should not receive thiomersal-containing products. Definitive data of doses at which developmental effects occur are not available. Moreover, revelation of subtle effects of toxicity needs long term observation of children.

The ethylmercury radical of the thiomersal molecule appears to be the prominent sensitiser. The prevalence of thiomersal hypersensitivity in mostly selected populations varies up to 18%, but higher figures have been reported. The overall exposure to thiomersal differs considerably between countries. In many cases a positive routine patch test to thiomersal should be considered an accidental finding without or, probably more accurately, with low clinical relevance.

In practice, some preventive measures can be taken with respect to thiomersal hypersensitivity. However, with regard to the debate on primary sensitisation during childhood and renewed attention for a reduction of children's exposure to mercury from all sources, the use of thiomersal should preferably be eliminated or at least be reduced. In 1999 the manufacturers of vaccines and immunoglobulins in the US and Europe were approached with this in mind. The potential toxicity in children seems to be of much more concern to them than the hidden sensitising properties of thiomersal.

In The Netherlands, unlike many other countries, the exposure to thiomersal from pharmaceutical sources has already been reduced. Replacement of thiomersal in all products should have a high priority in all countries.

Allergic and toxic reactions to medical or non-medical applications of inorganic and organic mercurials remain a focus of public and scientific attention. The organic mercurial thiomersal has been used as a preservative, particularly but not only in vaccines, for many decades and is a challenging example. Despite numerous reports on sensitisation to thiomersal for many decades, thiomersal is still being used.

The potential toxicity of thiomersal in children

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Full Communication

Effects of Glucan on Immunosuppressive Actions of Mercury

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ABSTRACT Global cycling of mercury results in the presence of mercury salts in the environment. The well-established negative effects of mercury on the immune system led us to the study whether natural immunomodulator glucan can overcome the immunosuppressive effects of mercury. Two types of mercury, thimerosal and mercury acetate, were administered in a dose of 2–8 mg/L of drinking water to mice. After 2 weeks, all mice exhibited profound suppression of both cellular (phagocytosis, natural killer cell activity, mitogen-induced proliferation, and expression of CD markers) and humoral (antibody formation and secretion of interleukin-6, interleukin-12, and interferon-γ) responses. The mice were then fed with a diet containing a standard dose of glucan. Our results showed that simultaneous treatment with mercury and glucan resulted in significantly lower immunotoxic effects of mercury, which suggests that glucans can be successfully used as a natural remedy of low-level exposure to mercury.

KEY WORDS: • ***glucan*** • ***immune system*** • ***immunosuppression*** • ***mercury*** • ***phagocytosis***

INTRODUCTION

FOR A LONG TIME, THIMEROSAL has been used as a wound disinfectant and a preservative in medical preparation, including human vaccines. Recently, concerns regarding the immunosuppressive effects of low-level exposure to mercury raised the question of thimerosal safety.[1,2] Thimerosal contains an organic ethylmercury with similar biological properties as the well-known immunotoxic methylmercury.[3,4] However, recent studies have shown that the effects on the immune system might be different.[5]

Additional studies showed that exposure to most mercury compounds, including mercuric chloride, resulted in cell toxicity[6] and immunosuppression[7] regardless of exposure duration.[8]

β1,3-Glucans are structurally complex homopolymers of glucose, usually isolated from yeast, fungi, wheat, and seaweed. β1,3-Glucan's role as a biologically active immunomodulator has been well documented for over 40 years. Interest in the immunomodulatory properties of polysaccharides was initially raised after experiments indicated that a crude yeast cell preparation stimulated macrophages via activation of the complement system.[9] Further work identified the immunomodulatory active component as β1,3-glucan.[10] Numerous studies (currently more than 4,000 publications) have subsequently shown that β1,3-glucans, either particulate or soluble, exhibit immunostimulating properties that include antibacterial and antitumor activities.[11,12] Studies showing the strong potential of glucans to help overcome immunosuppressive effects of factors, such as irradiation or chemotherapy,[13,14] led us to the hypothesis evaluated in this article. The aims of the present study are to compare immunosuppression caused by either organic (thimerosal) or inorganic (mercury acetate) mercury and to show if this suppression can be reversed by glucan.

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MATERIALS AND METHODS

Materials

RPMI 1640 medium, Iscoves's modified Dulbecco's medium, sodium citrate, antibiotics, sodium azide, thimerosal, mercury acetate, bovine serum albumin, Wright stain, *Limulus* lysate test E-TOXATE, Freund's adjuvant, ovalbumin, lipopolysaccharide, and concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT, USA). β-1,3-Glucan (#300) was purchased from Transfer Point (Columbia, SC, USA), NOW BETA glucan from NOW FOODS (Bloomingdale, IL, USA), Glucagel T from GraceLinc (Christchurch, New Zealand), and Epicor from Embria Health Sciences (Ankeny, IA, USA).

Animals

Female, 6–10-week-old BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All animal work was done according to the University of Louisville Institutional Animal Care and Use Committee protocol. Animals were sacrificed by CO_2 asphyxiation.

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IMMEDIATE COMMUNICATION

Activation of methionine synthase by insulin-like growth factor-1 and dopamine: a target for neurodevelopmental toxins and thimerosal

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Methylation events play a critical role in the ability of growth factors to promote normal development. Neurodevelopmental toxins, such as ethanol and heavy metals, interrupt growth factor signaling, raising the possibility that they might exert adverse effects on methylation. We found that insulin-like growth factor-1 (IGF-1)- and dopamine-stimulated methionine synthase (MS) activity and folate-dependent methylation of phospholipids in SH-SY5Y human neuroblastoma cells, via a PI3-kinase- and MAP-kinase-dependent mechanism. The stimulation of this pathway increased DNA methylation, while its inhibition increased methylation-sensitive gene expression. Ethanol potently interfered with IGF-1 activation of MS and blocked its effect on DNA methylation, whereas it did not inhibit the effects of dopamine. Metal ions potently affected IGF-1 and dopamine-stimulated MS activity, as well as folate-dependent phospholipid methylation: Cu^{2+} promoted enzyme activity and methylation, while Cu^{+}, Pb^{2+}, Hg^{2+} and Al^{3+} were inhibitory. The ethylmercury-containing preservative thimerosal inhibited both IGF-1- and dopamine-stimulated methylation with an IC_{50} of 1 nM and eliminated MS activity. Our findings outline a novel growth factor signaling pathway that regulates MS activity and thereby modulates methylation reactions, including DNA methylation. The potent inhibition of this pathway by ethanol, lead, mercury, aluminum and thimerosal suggests that it may be an important target of neurodevelopmental toxins.
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Keywords: autism; attention deficit hyperactivity disorder; P13-kinase; D4 dopamine receptor; DNA methylation; phospholipid methylation; lead; mercury

Introduction

Developmental disorders include a spectrum of neurological conditions characterized by deficits in attention, cognition and learning, frequently accompanied by abnormal behaviors. Severe deficits may be recognized at birth, but a failure to achieve standard milestones during initial years of life remains the primary basis of diagnosis in most cases. While the underlying cause(s) remains obscure for many developmental disorders, metabolic abnormalities involving purine synthesis (eg Lesch–Nyhan Syndrome and adenylsuccinate lyase deficiency)[1,2] or impaired methylation-dependent gene silencing and/or imprinting (Rett and Fragile-X syndromes)[3,4] suggest biochemical mechanisms that may be involved. The development disorders can also be caused by exposure to toxins (eg ethanol, in fetal alcohol syndrome; heavy metals, in lead poisoning),[5,6] although the precise mechanisms underlying their toxicity are not known. The recent increase in the incidence of autism has led to the speculation that environmental exposures including vaccine additives (ie aluminum and the ethylmercury-containing preservative thimerosal) might contribute to the triggering of this developmental disorder.[7]

Normal development is closely related to cellular differentiation, and growth factor-initiated signaling promotes differentiation of pluripotent cells.[8] Furthermore, altered patterns of DNA methylation and associated gene silencing underlie phenotypic differences between undifferentiated and differentiated cells.[9] Together, these observations suggest that growth factors promote cellular differentiation by producing effects on DNA methylation. This suggestion is reinforced by the observation that

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Research Article

Alternatively Spliced Methionine Synthase in SH-SY5Y Neuroblastoma Cells: Cobalamin and GSH Dependence and Inhibitory Effects of Neurotoxic Metals and Thimerosal

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The folate and cobalamin (Cbl-) dependent enzyme methionine synthase (MS) is highly sensitive to oxidation and its activity affects all methylation reactions. Recent studies have revealed alternative splicing of MS mRNA in human brain and patient-derived fibroblasts. Here we show that MS mRNA in SH-SY5Y human neuroblastoma cells is alternatively spliced, resulting in three primary protein species, thus providing a useful model to examine cofactor dependence of these variant enzymes. MS activity was dependent upon methylcobalamin (MeCbl) or the combination of hydroxocobalamin (OHCbl) and S-adenosylmethionine (SAM). OHCbl-based activity was eliminated by depletion of the antioxidant glutathione (GSH) but could be rescued by provision of either glutathionylcobalamin (GSCbl) or MeCbl. Pretreatment of cells with lead, arsenic, aluminum, mercury, or the ethylmercury-containing preservative thimerosal lowered GSH levels and inhibited MS activity in association with decreased uptake of cysteine, which is rate-limiting for GSH synthesis. Thimerosal treatment decreased cellular levels of GSCbl and MeCbl. These findings indicate that the alternatively spliced form of MS expressed in SH-SY5Y human neuronal cells is sensitive to inhibition by thimerosal and neurotoxic metals, and lower GSH levels contribute to their inhibitory action.

1. Introduction

MS is a multidomain enzyme which transfers a folate-derived methyl group to homocysteine (HCY), thereby creating methionine. The cobalamin (Cbl) cofactor of MS, its Cbl[I] form, directly participates in the transfer reaction by abstracting a folate-derived methyl group, temporarily creating methylcobalamin (MeCbl), and then transferring the methyl group to HCY [1]. However, if Cbl[I] oxidizes prior to MeCbl formation, enzyme activity is temporarily halted, increasing HCY diversion to the transsulfuration pathway and augmenting formation of cysteine, the rate-limiting metabolite for synthesis of the antioxidant GSH [2, 3]. In this manner Cbl serves as a redox sensor whose oxidation leads to increased antioxidant synthesis in proportion to cellular demand. MS inactivation is accompanied by decreased methylation activity, caused by lower levels of the methyl donor SAM and higher levels of the methylation inhibitor S-adenosylhomocysteine (SAH) [4]. Thus MS and Cbl link redox status to methylation status, including methylation of DNA and

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ORIGINAL ARTICLE

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Homozygous gene deletions of the glutathione *S*-transferases M1 and T1 are associated with thimerosal sensitization

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Abstract ***Objective:*** Thimerosal is an important preservative in vaccines and ophthalmologic preparations. The substance is known to be a type IV sensitizing agent. High sensitization rates were observed in contact-allergic patients and in health care workers who had been exposed to thimerosal-preserved vaccines. There is evidence for the involvement of the glutathione system in the metabolism of thimerosal or its decomposition products (organomercury alkyl compounds). Thus detoxification by polymorphically expressed glutathione *S*-transferases such as GSTT1 and GSTM1 might have a protective effect against sensitization by these substances. ***Methods:*** To address this question, a case control study was conducted, including 91 Central European individuals with a positive patch-test reaction to thimerosal. This population was compared with 169 healthy controls and additionally with 114 individuals affected by an allergy against para-substituted aryl compounds. The latter population was included in order to test whether possible associations were due to substance-specific effects, or were a general feature connected with type IV immunological diseases. Homozygous deletions of GSTT1 and GSTM1 were determined by polymerase chain reaction. ***Results:*** Glutathione *S*-transferase M1 deficiency was significantly more frequent among patients sensitized to thimerosal (65.9%, $P = 0.013$) compared with the healthy control group (49.1%) and the "para-compound" group (48%, $P = 0.034$). Glutathione *S*-transferase T1 deficiency in the thimerosal/mercury group (19.8%) was barely elevated versus healthy controls (16.0%) and the "para-compound" group (14.0%). The combined deletion (GSTT1−/GSTM1−) was markedly more frequent among thimerosal-sensitized patients than in healthy controls (17.6% vs. 6.5%, $P = 0.0093$) and in the "para-compound" group (17.6% vs. 6.1%, $P = 0.014$), revealing a synergistic effect of these enzyme deficiencies (healthy controls vs. thimerosal GSTM1 negative individuals, OR = 2.0 [CI = 1.2–3.4], GSTT1−, OR = 1.2 [CI = 0.70–2.1], GSTM1/T1−, OR = 3.1 [CI = 1.4–6.5]). ***Conclusions:*** Since the glutathione-dependent system was repeatedly shown to be involved in the metabolism of thimerosal decomposition products, the observed association may be of functional relevance.

Key words Glutathione *S*-transferase · Polymorphism · Thimerosal

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Introduction

The predisposition to acquire a sensitization towards certain contact-allergens was proposed to be heritable. This proposal was based mainly on family and twin studies (reviewed by Menné and Holm 1986). Among

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GENOTOXICITY

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Thimerosal induces micronuclei in the cytochalasin B block micronucleus test with human lymphocytes

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Abstract Thimerosal is a widely used preservative in health care products, especially in vaccines. Due to possible adverse health effects, investigations on its metabolism and toxicity are urgently needed. An in vivo study on chronic toxicity of thimerosal in rats was inconclusive and reports on genotoxic effects in various in vitro systems were contradictory. Therefore, we reinvestigated thimerosal in the cytochalasin B block micronucleus test. Glutathione S-transferases were proposed to be involved in the detoxification of thimerosal or its decomposition products. Since the outcome of genotoxicity studies can be dependent on the metabolic competence of the cells used, we were additionally interested whether polymorphisms of glutathione S-transferases (GSTM1, GSTT1, or GSTP1) may influence the results of the micronucleus test with primary human lymphocytes. Blood samples of six healthy donors of different glutathione S-transferase genotypes were included in the study. At least two independent experiments were performed for each blood donor. Significant induction of micronuclei was seen at concentrations between 0.05–0.5 μg/ml in 14 out of 16 experiments. Thus, genotoxic effects were seen even at concentrations which can occur at the injection site. Toxicity and toxicity-related elevation of micronuclei was seen at and above 0.6 μg/ml thimerosal. Marked individual and intraindividual variations in the in vitro response to thimerosal among the different blood donors occurred. However, there was no association observed with any of the glutathione S-transferase polymorphism investigated. In conclusion, thimerosal is genotoxic in the cytochalasin B block micronucleus test with human lymphocytes. These data raise some concern on the widespread use of thimerosal.

Keywords Thimerosal · Cytokinesis-block micronucleus assay · Glutathione S-transferase

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Introduction

Thimerosal {sodium ethyl[2-mercaptobenzoato(2–)-*O*, *S*]mercurate(1–), CAS 54-64-8}, is used as a preservative in medical products, especially in hepatitis B vaccines. The discussion on toxic effects of thimerosal is mainly focussed on its mercury content (Ball et al. 2001). In addition, the substance is known to be a contact sensitiser (Schnuch et al. 1998). Possible carcinogenic effects were investigated in one study on the chronic toxicity of thimerosal in Fischer 344 rats (Mason et al. 1971). However, this study does not meet the requirements of the current guidelines and does not rule out a possible carcinogenic effect of thimerosal.

In addition, there were reports on genotoxic effects of thimerosal in vivo. A weak but significant increase in micronuclei and chromosome aberrations was seen in male Swiss CD-1 mice at doses between 10 and 20 mg/kg (Marrazzini et al. 1994); another study using male and female (102/E1×C3H/E1)F_1 mice and Swiss albino mice reported negative results (Adler et al. 1991).

Reports on genotoxic effects in in vitro systems were contradictory. According to the acceptance criteria outlined by the GUM (German Section of the European Environmental Mutagen Society) working group on the evaluation of published data of the in vitro micronucleus test, only two valid reports on the effects of thimerosal in this test system were available (Miller et al. 1998). A weak but significant induction of micronuclei was found at concentrations between 0.01 and 0.16 μg/ml in two out of three experiments with human lymphocytes from two donors (Migliore and Nieri 1991). A significant elevation of micronuclei in V79 cells was induced by

Case report

A generalized reaction to thimerosal from an influenza vaccine

Mary Lee-Wong, MD*; David Resnick, MD†; and Kaman Chong, DO*

Background: Thimerosal is a preservative commonly used in ophthalmic solutions, otic drops, and vaccines because of its bactericidal property.

Objective: To report a case of a generalized reaction to thimerosal in a patient who received an influenza vaccine.

Methods: We describe a patient who developed a generalized maculopapular eruption after receiving a thimerosal-containing influenza vaccine. Patch testing was performed to determine if there was an allergy to thimerosal.

Results: Patch testing confirmed a T-cell–mediated sensitivity to thimerosal.

Conclusions: Physicians need to be aware that thimerosal is found in many products, including vaccinations. Clinicians should also be aware that allergic reactions occur with exposure to thimerosal even in vaccines. To our knowledge, this is the first case report in the literature of a generalized reaction to thimerosal from an influenza vaccine.

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INTRODUCTION

Thimerosal is a mercury derivative preservative that has been used since the 1930s. It can be found in cosmetics, ophthalmic solutions, otic products, adrenal cortex or testosterone injections, antivenins, immunoglobulins, nasal sprays, and vaccines.[1,2] It is effective in preventing bacterial contamination in the aforementioned products and is used especially in multidose containers of vaccine.[3] The US Public Health Service and American Academy of Pediatrics set a goal to reduce or eliminate thimerosal in vaccines to minimize mercury exposure in July 1999.[4,5] This precautionary measure was aimed especially at children, since they are more susceptible to the toxic effects of mercury. Currently, all recommended vaccines for children are free of thimerosal; however, thimerosal is still used in adult vaccines, including tetanus, influenza, pneumococcal vaccines, and some hepatitis B.[6] Herein, we report a case of a generalized reaction to thimerosal in a patient who received an influenza vaccine.

CASE REPORT

A 39-year-old white woman developed pruritus and a rash on all 4 extremities 8 hours after receiving an influenza vaccine. The patient received the injection in the right deltoid muscle. She initially experienced pruritus on the right arm that spread to the left arm, both legs, and upper chest. She had been taking montelukast sodium, fexofenadine hydrochloride–pseudoephedrine hydrochloride, mometasone furoate monohydrate, salmeterol xinafoate–fluticasone propionate (inhalation powder), escitalopram oxalate, and lisinopril-hydrochlorothiazide. She had been taking all of these medications for more than 3 months. She had not taken any other medication, vitamins, or herbs. The patient had received the influenza vaccine each year for the past 6 years without a reaction. Of significance is the fact that the patient developed a rash on her eyelids 10 years previously from a thimerosal-containing contact lens solution.

Her physical examination revealed an erythematous, maculopapular eruption on all 4 extremities and the torso. There were neither hives nor mucosal lesions. Her medical history was significant for hypertension, asthma, allergic rhinitis, and conjunctivitis. She developed hives after contact exposure to horses. There was a history of lip swelling after ingestion of honey dew, and she experienced a generalized pruritic rash after ingestion of raspberries as a child. The patient denied exposure to poison ivy or an egg allergy. She worked as a clerk and denied any exposure to mercury-related products or use of cosmetics.

The patient's rash spread from her lower extremities up to her buttocks and persisted for 2 weeks before she was referred to our allergy clinic. She was treated with a 5-day course of oral prednisone and a sedating antihistamine to decrease the pruritus. The rash completely resolved after 4 days. The patient returned 4 weeks later, and patch testing was performed (T.R.U.E. Test; Glaxo Pharmaceuticals, Research Triangle Park, NC). The only allergen that induced an allergic response was thimerosal (Figs 1 through 3).

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Thiol-Modulated Mechanisms of the Cytotoxicity of Thimerosal and Inhibition of DNA Topoisomerase IIα

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Thimerosal is an organic mercury compound that is widely used as a preservative in vaccines and other solution formulations. The use of thimerosal has caused concern about its ability to cause neurological abnormalities due to mercury accumulation during a normal schedule of childhood vaccinations. While the chemistry and the biological effects of methylmercury have been well-studied, those of thimerosal have not. Thimerosal reacted rapidly with cysteine, GSH, human serum albumin, and single-stranded DNA to form ethylmercury adducts that were detectable by mass spectrometry. These results indicated that thimerosal would be quickly metabolized in vivo because of its reactions with protein and nonprotein thiols. Thimerosal also potently inhibited the decatenation activity of DNA topoisomerase IIα, likely through reaction with critical free cysteine thiol groups. Thimerosal, however, did not act as a topoisomerase II poison and the lack of cross-resistance with a K562 cell line with a decreased level of topoisomerase IIα (K/VP.5 cells) suggested that inhibition of topoisomerase IIα was not a significant mechanism for the inhibition of cell growth. Depletion of intracellular GSH with buthionine sulfoximine treatment greatly increased the K562 cell growth inhibitory effects of thimerosal, which showed that intracellular glutathione had a major role in protecting cells from thimerosal. Pretreatment of thimerosal with glutathione did not, however, change its K562 cell growth inhibitory effects, a result consistent with the rapid exchange of the ethylmercury adduct among various thiol-containing cellular reactants. Thimerosal-induced single and double strand breaks in K562 cells were consistent with a rapid induction of apoptosis. In conclusion, these studies have elucidated some of the chemistry and biological activities of the interaction of thimerosal with topoisomerase IIα and protein and nonprotein thiols and with DNA.

Introduction

Thimerosal (Figure 1) is an organic mercury compound with bactericidal and fungicidal properties that is widely used as a preservative in multiuse vials of vaccines, ophthalmic, otic, nasal, and topical products (*1–3*). There has been a public perception that thimerosal use in vaccines is unsafe after suggestions that it caused a predisposition to autism in children (*1, 4*). However, recent epidemiological studies have not supported this hypothesis (*4*). On the basis of the risk assessment assumption that the dose–effect and dose–response relationships of ethylmercury, the presumed metabolite of thimerosal, and methylmercury were the same, thimerosal was removed from most pediatric vaccines in the United States in 2001 (*1, 3*). Prior to 2001, by 18 months of age, a child in the United States undergoing a routine schedule of immunizations would have received a cumulative dose of 200 μg of mercury (*3*). The fact that the cumulative exposure to mercury from thimerosal in infants undergoing immunization during the first 6 months of life could exceed U.S. Environmental Protection Agency guidelines provided impetus for the removal of thimerosal from pediatric vaccines (*3*).

Much of what is known about chronic low-dose human methymercury toxicity causing neurologic abnormalities comes from poisoning episodes and environmental exposure (*1, 3*). Far less is known about the effects of thimerosal or its presumed metabolite, ethylmercury (*1, 3, 5, 6*). The initial distribution of ethylmercury in neonatal mice is similar to that of methylmercury, but they differ sharply in their tissue deposition and their metabolism to Hg^{2+} (*4*). This suggests that the data on methylmercury may not be suitable for risk assessment for thimerosal (*1, 5*). Methylmercury reacts rapidly with and has a very high affinity for protein and nonprotein thiols (*1, 78*), and ethylmercury is likely similar in this regard.

Thus, to elucidate some of the basic chemistry and biochemistry of thimerosal, the reactions of thimerosal with nonprotein and protein thiols and the cellular effects of thimerosal have been studied. While the reaction of thimerosal with thiols has been assumed to be an exchange reaction to yield an ethylmercury-thiol adduct (Figure 1), this does not seem to have been shown. In this study, we showed by MS that thimerosal undergoes an exchange reaction with cysteine, GSH, and human serum albumin (HSA)[1] (Figure 1) and forms an ethylmercury adduct with single-stranded DNA.

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[1] Abbreviations: Annexin V-FITC, annexin V-fluorescein isothiocyanate conjugate; 6-mer DNA, DNA with the sequence 5′-CACGTG-3′; 20-mer DNA, self-complementary hairpin DNA with the sequence 5′-TAT-GATATTTTTATATCATA-3′; BSO, buthionine sulfoximine; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HSA, human serum albumin; IC_{50}, 50% inhibitory concentration; kDNA, kinetoplast DNA; thimerosal-DNA, thimerosal-treated and washed kDNA; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; OTC, (−)-2-oxo-4-thiazolidinecarboxylic acid.

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INTERMINGLED MODULATORY AND NEUROTOXIC EFFECTS OF THIMEROSAL AND MERCURIC IONS ON ELECTROPHYSIOLOGICAL RESPONSES TO GABA AND NMDA IN HIPPOCAMPAL NEURONS

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The organomercurial, thimerosal, is at the center of medical controversy as a suspected factor contributing to neurodevelopmental disorders in children. Many neurotoxic effects of thimerosal have been described, but its interaction with principal excitatory and inhibitory neurotransmiter systems is not known. We examined, using electrophysiological recordings, thimerosal effects on GABA and NMDA-evoked currents in cultured hippocampal neurons. After brief (3 to 10 min) exposure to thimerosal at concentrations up to 100 μM, there was no significant effect on GABA or NMDA-evoked currents. However, following exposure for 60-90 min to 1 or 10 μM thimerosal, there was a significant decrease in NMDA-induced currents ($p<0.05$) and GABAergic currents ($p<0.05$). Thimerosal was also neurotoxic, damaging a significant proportion of neurons after 60-90 min exposure; recordings were always conducted in the healthiest looking neurons. Mercuric chloride, at concentrations 1 μM and above, was even more toxic, killing a large proportion of cells after just a few minutes of exposure. Recordings from a few sturdy cells revealed that micromolar mercuric chloride markedly potentiated the GABAergic currents ($p<0.05$), but reduced NMDA-evoked currents ($p<0.05$). The results reveal complex interactions of thimerosal and mercuric ions with the $GABA_A$ and NMDA receptors. Mercuric chloride act rapidly, decreasing electrophysiological responses to NMDA but enhancing responses to GABA, while thimerosal works slowly, reducing both NMDA and GABA responses. The neurotoxic effects of both mercurials are interwoven with their modulatory actions on $GABA_A$ and NMDA receptors, which most likely involve binding to these macromolecules.

Key words: *$GABA_A$ receptors, neurotoxicity, NMDA receptors, patch-clamp, thimerosal, mercuric ions, hippocampal neurons*

INTRODUCTION

Thimerosal (THIM), an organomercurial containing approximately 49% of mercury by weight, has been added for decades to medicinal products, including pediatric vaccines, without being sufficiently tested for its safety. This is surprising in view of the fact that all mercurials are highly toxic, particularly to developing organisms. In the past decade concerns emerged about the possibility that THIM from vaccines might contribute to certain neurodevelopmental disorders in children, which prompted its recent removal from most pediatric vaccines in the Western countries (7, 19). Unfortunately, it is still added to pediatric vaccines in less developed countries, including Poland, potentially damaging the health of children.

THIM is metabolized in the body to ethyl mercury (EtHg) and subsequently to inorganic mercury forms, which accumulate in tissues of vital organs, including the brain (22). Information about neurochemical and neurotoxic effects of THIM is still limited, but the existing data indicate that in pharmacodynamics and toxicity THIM/EtHg does not differ significantly from methyl mercury (MeHg), which has been studied more extensively, although these compounds differ somewhat in pharmacokinetics (8).

Several studies documented that the neurotoxic effects of mercurials involve glutamate-mediated excitotoxicty, due to their ability to inhibit uptake of glutamate in astrocytes, resulting in an increase of the extracellular level of this excitatory amino acid (1, 4, 14). Excessive synaptic activity of glutamate may lead to excitotoxicity. Mercurials may interact as well with the glutamate receptors. MeHg has been shown to alter gene expression for the NMDA receptors (16) and to inhibit NMDA receptor binding *in vitro* (23), but in electrophysiological recordings both MeHg and $HgCl_2$ were without apparent rapid modulatory effect on the NMDA-induced currents in neurons (25). Equally ambiguous are the effects of mercurials on function of $GABA_A$ receptors. Electrophysiological studies demonstrated that both MeHg and inorganic Hg interact with neuronal $GABA_A$ receptors, albeit in opposite directions, as $HgCl_2$ potentiated the GABAergic currents, whereas MeHg decreased them (11, 20). An *in vivo* study showed an increased number of benzodiazepine receptors in rat brain, three days after acute MeHg administration (9).

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BASIC SCIENCE

Cytoprotective effect of hyaluronic acid and hydroxypropyl methylcellulose against DNA damage induced by thimerosal in Chang conjunctival cells

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Abstract
Background To investigate genotoxicity of the preservative thimerosal (Thi), and the cytoprotective and antioxidant effects of hyaluronic Acid (HA) and hydroxypropyl methylcellulose (HPMC) on Chang conjunctival cells.
Method Cells were divided into three groups. One group was exposed to Thi at various concentrations (0.00001 %~0.001 %) for 30 min; the other two groups were pretreated with 0.3 % HA or 0.3 % HPMC for 30 min before the Thi exposure. After cell viability was evaluated, alkaline comet assay and detection of the phosphorylated form of the histone variant H2AX (γH2AX) foci were used to determine DNA damage. Reactive oxygen species (ROS) production was assessed by the fluorescent probe, 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA).
Results A significant change of cell viability was observed after exposure to 0.001 % Thi for 30 min. DNA single- and double-strand breaks were significantly increased in a dose-dependent manner with Thi exposure. In addition, intracellular ROS induced by Thi was dose-dependent, except at 0.001 % less ROS was induced than at 0.0005 %. However, cells pretreated with 0.3 % HA or 0.3 % HPMC showed significantly increased cell survival, decreased DNA damage, and decreased ROS production compared to cells exposed to Thi alone. Pretreatment with 0.3 % HA was found to be even more protective than 0.3 % HPMC.
Conclusion Thi can induce DNA damage in human conjunctival epithelial cells, probably due to oxidative stress. HA and HPMC are protective agents that have antioxidant properties and can decrease DNA damage induced by Thi. Pretreatment of 0.3 % HA may be more protective of the ocular surface than 0.3 % HPMC.

Keywords Thimerosal · Hyaluronic acid · Hydroxypropyl methylcellulose · DNA damage · Cytoprotective · Chang conjunctival cells · ROS · γH2AX foci

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Introduction

Thimerosal (Thi) is an organomercurial compound that is widely used as an antiseptic/antifungal agent in cosmetics, pharmaceutical products, and vaccines. It is also widely used in ophthalmic preparations, mainly drugs and contact lens solutions. Its usual concentration in ocular drugs ranges from 0.001 % to 0.004 %. Recently, deleterious effects on the ocular surface and corneal endothelium have been observed with long-term use of topical drugs containing Thi. Thi might cause structural and functional damage to the endothelium with prolonged, direct exposure [1]. In addition, evidence suggests that Thi might be responsible for delayed hypersensitivity, which can cause conjunctival hyperemia and corneal infiltrates [2].

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Thimerosal induces neuronal cell apoptosis by causing cytochrome *c* and apoptosis-inducing factor release from mitochondria

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Abstract. There is a worldwide increasing concern over the neurological risks of thimerosal (ethylmercury thiosalicylate) which is an organic mercury compound that is commonly used as an antimicrobial preservative. In this study, we show that thimerosal, at nanomolar concentrations, induces neuronal cell death through the mitochondrial pathway. Thimerosal, in a concentration- and time-dependent manner, decreased cell viability as assessed by calcein-ethidium staining and caused apoptosis detected by Hoechst 33258 dye. Thimerosal-induced apoptosis was associated with depolarization of mitochondrial membrane, generation of reactive oxygen species, and release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria to cytosol. Although thimerosal did not affect cellular expression of Bax at the protein level, we observed translocation of Bax from cytosol to mitochondria. Finally, caspase-9 and caspase-3 were activated in the absence of caspase-8 activation. Our data suggest that thimerosal causes apoptosis in neuroblastoma cells by changing the mitochondrial microenvironment.

Introduction

Apoptosis is a physiological form of cell suicide that plays a role in embryogenesis, metamorphosis, cellular homeostasis, and as a defensive mechanism to remove infected, mutated, or damaged cells (1,2). Apoptosis is characterized by loss of cellular contact with the matrix, cytoplasmic contraction, chromatin condensation, plasma membrane blebbing, and DNA fragmentation into large and small oligosomes. Apoptosis takes place through the death receptors (3-6) and/or involvement of the mitochondrial pathway (7,8), with molecular and biochemical steps leading to the activation of common effector or executioner cysteine proteases, the caspases resulting in the cleavage of a number of nuclear and cytoplasmic substrates that culminate in apoptosis. Because of the role of apoptosis in cellular homeostasis, disorders of apoptosis result in either the accumulation of abnormal cells, leading to cancer and autoimmunity, or in the loss of cells, leading to immunodeficiency and neurodegenerative diseases (9).

There is an increasing concern throughout the world about the risks of environmental exposure to mercury, which is ubiquitously found in fish, dental amalgams, and in preservatives (10-17). One of the mercury compounds that has recently come to public attention, because of its wide usage as an antibacterial and antifungal preservative in biomedical products and vaccines, is thimerosal (10-12). Thimerosal (ethylmercury salicylate) contains 49.6% mercury by weight and is metabolized to ethylmercury and thiosalicylate (15). In the body, ethylmercury readily passes through cellular membranes and concentrates in vital tissue and organs, including the central nervous system where it can exert its toxicity over a prolonged period of time (12,16). However, the effects of thimerosal on neuronal cell functions, especially on apoptosis, are poorly understood and largely unexplored.

During the last decade, there has been a better understanding of the role of mitochondria in cell death (7,8,18-20). A number of stimuli, including chemotherapeutic agents, radiation, and stress molecules, e.g. reactive oxygen and reactive nitrogen species, appear to mediate apoptosis via mitochondrial pathway. It is known that some mercury derivatives, such as methylmercury, induce oxidative stress (21-24). In this study, in order to shed light on the claimed link between thimerosal and neurotoxicity, we investigated the effect of thimerosal on cell viability, mitochondrial microenvironment and downstream steps of apoptosis in neuronal cells. We observed that thimerosal, in nanomolar concentrations, caused cell death/apoptosis which was mediated through the mitochondria evidenced by cytochrome *c* and AIF release and activation of caspase-9.

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Abbreviations: AIF, apoptosis-inducing factor; $\Delta\Psi$, mitochondrial membrane potential; DHE, dihydroethidium; EthD-1, ethidium; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species

Key words: thimerosal, mitochondria, cytochrome *c*, apoptosis-inducing factor, bax

Review

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Mercury Promotes Catecholamines Which Potentiate Mercurial Autoimmunity and Vasodilation: Implications for Inositol 1,4,5-Triphosphate 3-Kinase C Susceptibility in Kawasaki Syndrome

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Previously, we reviewed biological evidence that mercury could induce autoimmunity and coronary arterial wall relaxation as observed in Kawasaki syndrome (KS) through its effects on calcium signaling, and that inositol 1,4,5-triphosphate 3-kinase C (ITPKC) susceptibility in KS would predispose patients to mercury by increasing Ca^{2+} release. Hg^{2+} sensitizes inositol 1,4,5-triphosphate (IP3) receptors at low doses, which release Ca^{2+} from intracellular stores in the sarcoplasmic reticulum, resulting in delayed, repetitive calcium influx. ITPKC prevents IP3 from triggering IP3 receptors to release calcium by converting IP3 to inositol 1,3,4,5-tetrakisphosphate. Defective IP3 phosphorylation resulting from reduced genetic expressions of ITPKC in KS would promote IP3, which increases Ca^{2+} release. Hg^{2+} increases catecholamine levels through the inhibition of S-adenosylmethionine and subsequently catechol-O-methyltransferase (COMT), while a single nucleotide polymorphism of the COMT gene (rs769224) was recently found to be significantly associated with the development of coronary artery lesions in KS. Accumulation of norepinephrine or epinephrine would potentiate Hg^{2+}-induced calcium influx by increasing IP3 production and increasing the permeability of cardiac sarcolemma to Ca^{2+}. Norepinephrine and epinephrine also promote the secretion of atrial natriuretic peptide, a potent vasodilator that suppresses the release of vasoconstrictors. Elevated catecholamine levels can induce hypertension and tachycardia, while increased arterial pressure and a rapid heart rate would promote arterial vasodilation and subsequent fatal thromboses, particularly in tandem. Genetic risk factors may explain why only a susceptible subset of children develops KS although mercury exposure from methylmercury in fish or thimerosal in pediatric vaccines is nearly ubiquitous. During the infantile acrodynia epidemic, only 1 in 500 children developed acrodynia whereas mercury exposure was very common due to the use of teething powders. This hypothesis mirrors the leading theory for KS in which a widespread infection only induces KS in susceptible children. Acrodynia can mimic the clinical picture of KS, leading to its inclusion in the differential diagnosis for KS. Catecholamine levels are often elevated in acrodynia and may also play a role in KS. We conclude that KS may be the acute febrile form of acrodynia. **(Korean Circ J 2013;43:581-591)**

KEY WORDS: Kawasaki syndrome; Catecholamines; Mercury; Autoimmunity.

Introduction

Kawasaki syndrome (KS) is an acute febrile illness which predominately occurs in young children under 5 years of age (75-80%) while it is exceptional in adults (<1%). The clinical picture consists of a persistent, erratically spiking-high fever ranging from 38° to 40°C (101° to 104°F) which is resistant to antipyretics and antibiotics. In addition to the fever, four out of five of the following principle features are required for diagnosis: 1) a polymorphous rash; 2) conjunctival injection; 3) bright red, swollen extremities with subsequent desquamation typically during the second or third week; 4) oral changes which include bright red fissured lips, oropharyngitis, st-

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Cell death and cytotoxic effects in YAC-1 lymphoma cells following exposure to various forms of mercury

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Abstract

The effects of 1 min–4 h exposures to four Hg compounds (mercuric chloride [$HgCl_2$], methyl mercuric chloride [CH_3HgCl], *p*-chloromercuribenzoate [p-CMB] and thimerosal [TMS; ethylmercurithiosalicylate]) on cell death, microtubules, actin, CD3 receptor expression, protein tyrosine phosphorylation (PTyr-P) and intracellular calcium ($[Ca^{2+}]_i$) levels were investigated in YAC-1 lympoma cells using flow cytometry. YOPRO-1 (YP) and propidium iodide (PI) dye uptake indicated all forms of Hg tested were toxic at concentrations ranging from 25.8–48.4 μM, with two distinct patterns of effects. Early apoptosis was prolonged for CH_3HgCl- and TMS-treated cells, with more than 50% remaining YP^+/PI^- after 4 h. Both CH_3HgCl and TMS induced complete loss of β-tubulin fluorescence, indicative of microtubule depolymerization and inhibition of tubulin synthesis and/or β-tubulin degradation, while F-actin fluorescence diminished to a lesser degree and only after loss β-tubulin. CH_3HgCl and TMS induced an almost immediate two-fold increase in CD3 fluorescence, with levels returning to baseline within minutes. With continued exposure, CD3 fluorescence was reduced to approximately 50% of baseline values. Both compounds also increased PTyr-P two- to three-fold immediately, with levels returning to baseline at 4 h. Similarly, two- to three-fold increases in $[Ca^{2+}]_i$ were noted after 1 min exposure. $[Ca^{2+}]_i$ increased progressively, reaching levels five- to eight-fold greater than control values. In contrast, dye uptake was delayed with $HgCl_2$ and p-CMB, although cell death proceeded rapidly, with almost all non-viable cells being late apoptotic (YP^+/PI^+) by 4 h. p-CMB produced early reductions in F-actin, and after 4 h, complete loss of F-actin with only partial reduction of total β-tubulin was seen with both p-CMB and $HgCl_2$. $HgCl_2$ reduced CD3 expression and PTyr-P slightly within minutes, while p-CMB produced similar effects on CD3 only at 4 h, at which time PTyr-P was increased two- to three-fold. Both compounds increased $[Ca^{2+}]_i$ within minutes, though levels remained under twice the baseline concentration after 15 min exposure. With continued exposure, $[Ca^{2+}]_i$ increased to levels two- to five-fold greater than control values. These findings indicate the two groups of Hg compounds may induce cell death by distinct pathways, reflecting interactions with different cellular targets leading to cell death.

Keywords: Mercuric chloride; Methyl mercuric chloride; *p*-Chloromercuribenzoate; Thimerosal; Cytotoxicity; YAC-1 lymphoma

1. Introduction

Many of the proposed targets for mercury (Hg) toxicity are also components of the localized supramolecular activation complex (SMAC) or 'immunological synapse' formed between antigen-presenting cells (APCs) and responding lymphocytes (Monks et al., 1998). But, while antigenic stimuli are restricted to the localized SMAC, Hg may interact non-specifically with thiol (–SH) groups throughout the cell. Non-localized Hg impacts may mimic antigen-mediated signaling at certain concentrations, but the effects are likely not

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PORPHYRINURIA IN KOREAN CHILDREN WITH AUTISM: CORRELATION WITH OXIDATIVE STRESS

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder believed to be associated with heavy metal exposure, especially mercury (Hg), and is characterized by disturbances in metal elimination. Various studies correlated elevated heavy metal body burden with ASD diagnoses as evidenced by increased urinary porphyrin levels in patients. Urinary porphyrins were also determined in Korean patients diagnosed with ASD (n = 65) who visited AK Eastern Medicinal Clinic in Kangnam-gu, Seoul, from June 2007 to September 2008, compared to controls (n = 9) residing in the same area, by means of Metametrix (CLIA-approved) laboratory testing. Further, urinary organic acids as indicators of hepatic detoxification/oxidative stress were also analyzed among patients diagnosed with ASD. Significant increases were found in patients diagnosed with ASD for proporphyrins, pentacarboxyporphyrin, precoproporphyrin, coproporphyrins, and total porphyrins. Significant correlations were observed between hepatic detoxification/oxidative stress markers and urinary porphyrins. In agreement with published data, the present results demonstrated that measurement of porphyrins serves as a reliable tool for diagnosis of heavy metal involvement in ASD.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social skills, cognitive ability, learning of stereotypical behaviors, bidirectional communication, overall sensory abilities, and gross and fine motor control and is manifested by abnormal behaviors (Eigsti & Shapiro, 2003; Werner & Dawson, 2005). While there is no consensus on the underlying cause of the disease, the factor of genetic inheritance is well known to play a critical role in disease development. Further, numerous epidemiological studies established a role for mercury (Hg) poisoning to be implicated in the development of ASD (Counter et al., 2002; Holmes et al., 2003, Geier & Geier, 2005, 2006a, 2006c; Geier et al., 2009c, Palmer et al., 2006, 2009; Windham et al., 2006; Young et al., 2008). Subjects exposed to Hg poisoning experience immune dysfunction and impairments in sensory, motor, and overall neuronal system, and exhibit abnormal behavior, which resemble characteristic symptoms of patients diagnosed with ASD (Faustman et al., 2000, Bernard et al., 2001, 2002; Redwood et al., 2001; Sweet & Zelikoff, 2001; Blaxill et al., 2004; Geier et al., 2008).

Many patients diagnosed with ASD carry single-nucleotide polymorphisms in genes involved in pathways for glutathione (GSH) synthesis, resulting in reduced activity of these pathways (Buyske et al., 2006; James et al., 2006). Glutathione is critical for detoxification of Hg, and GSH was found to be significantly decreased among patients diagnosed with ASD (Geier et al., 2009a).

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Thimerosal exposure in infants and neurodevelopmental disorders: An assessment of computerized medical records in the Vaccine Safety Datalink

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Abstract

The study evaluated possible associations between neurodevelopmental disorders (NDs) and exposure to mercury (Hg) from Thimerosal-containing vaccines (TCVs) by examining the automated Vaccine Safety Datalink (VSD). A total of 278,624 subjects were identified in birth cohorts from 1990–1996 that had received their first oral polio vaccination by 3 months of age in the VSD. The birth cohort prevalence rate of medically diagnosed International Classification of Disease, 9th revision (ICD-9) specific NDs and control outcomes were calculated. Exposures to Hg from TCVs were calculated by birth cohort for specific exposure windows from birth-7 months and birth-13 months of age. Poisson regression analysis was used to model the association between the prevalence of outcomes and Hg doses from TCVs. Consistent significantly increased rate ratios were observed for autism, autism spectrum disorders, tics, attention deficit disorder, and emotional disturbances with Hg exposure from TCVs. By contrast, none of the control outcomes had significantly increased rate ratios with Hg exposure from TCVs. Routine childhood vaccination should be continued to help reduce the morbidity and mortality associated with infectious diseases, but efforts should be undertaken to remove Hg from vaccines. Additional studies should be conducted to further evaluate the relationship between Hg exposure and NDs.

Keywords: ADD; ADHD autistic disorder; ASD; Ethylmercury; Merthiolate; Thiomersal

1. Introduction

In the last few decades, vaccines—one of the greatest breakthroughs in health sciences—have helped to accomplish striking reductions of infection and disease worldwide [1]. From the 1930s through the early 2000s, many routinely administered childhood vaccines in the United States contained Thimerosal [2]. Thimerosal is an organic mercury-containing compound that is 49.55% mercury (Hg) by weight, and initially metabolized to ethylmercury compounds and thiosalicylate [3].

The American Academy of Pediatrics and the US Public Health Service in 1999 [4] published a joint statement that urged "all government agencies to work rapidly toward reducing children's exposure to mercury from all sources." The statement recommended that Thimerosal be removed from vaccines as soon as possible as part of this overall process. Between 1999 and 2001, many vaccines recommended for children ≤6 years of age were made available in

Abbreviations: ADD, Attention Deficit Disorder; ADHD, Attention Deficit Hyperactivity Disorder; ASD, Autism Spectrum Disorder; IRB, Institutional Review Board; ICD-9, International Classification of Disease, 9th revision; Hg, Mercury; μg, micrograms; NDs, neurodevelopmental disorders; TCVs, Thimerosal-containing vaccines; US Agency for Toxic Substances and Disease Registry; CDC, US Centers for Disease Control and Prevention; EPA, US Environmental Protection Agency; FDA, US Food and Drug Administration; VAERS, Vaccine Adverse Event Reporting System; VSD, Vaccine Safety Datalink.

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Thimerosal distribution and metabolism in neonatal mice: comparison with methyl mercury

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ABSTRACT: Thimerosal, which releases the ethyl mercury radical as the active species, has been used as a preservative in many currently marketed vaccines throughout the world. Because of concerns that its toxicity could be similar to that of methyl mercury, it is no longer incorporated in many vaccines in the United States. There are reasons to believe, however, that the disposition and toxicity of ethyl mercury compounds, including thimerosal, may differ substantially from those of the methyl form. The current study sought to compare, in neonatal mice, the tissue concentrations, disposition and metabolism of thimerosal with that of methyl mercury. ICR mice were given single intramuscular injections of thimerosal or methyl mercury (1.4 mg Hg kg^{-1}) on postnatal day 10 (PND 10). Tissue samples were collected daily on PND 11–14. Most analysed tissues demonstrated different patterns of tissue distribution and a different rate of mercury decomposition. The mean organic mercury in the brain and kidneys was significantly lower in mice treated with thimerosal than in the methyl mercury-treated group. In the brain, thimerosal-exposed mice showed a steady decrease of organic mercury levels following the initial peak, whereas in the methyl mercury-exposed mice, concentrations peaked on day 2 after exposure. In the kidneys, thimerosal-exposed mice retained significantly higher inorganic mercury levels than methyl mercury-treated mice. In the liver both organic and inorganic mercury concentrations were significantly higher in thimerosal-exposed mice than in the methyl mercury group. Ethyl mercury was incorporated into growing hair in a similar manner to methyl mercury. The data showing significant kinetic differences in tissue distribution and metabolism of mercury species challenge the assumption that ethyl mercury is toxicologically identical to methyl mercury.

KEY WORDS: thimerosal; methyl mercury; distribution; metabolism; mice; neonatal exposure

Introduction

Thimerosal is an organic mercurial compound that has been used for over 60 years as a preservative in vaccines and other pharmaceutical products to prevent unwanted bacterial and fungal growth (U.S. Pharmacopeia, 1999). Thimerosal contains 49.6% mercury by weight and breaks down in the body to ethyl mercury and thiosalicylate (Tan and Parkin, 2000).

Public interest in the thimerosal content of vaccines began to develop after a report asserting that thimerosal in hepatitis B immunoglobulin caused severe ethyl mercury intoxication (Lowell *et al*., 1996). Because of scarcity of toxicological information on ethyl mercury exposure in humans, methyl mercury standards were used as surrogates for ethyl mercury based on the structural similarity of the two mercury species. In 1995, the Environmental Protection Agency introduced stricter guidelines for methyl mercury based on prenatal exposure (EPA, 1997). Public debate and the Food and Drug Administration (FDA) preliminary risk assessment prompted a joint statement of the American Academy of Pediatrics and the US Public Health Service calling for the removal of thimerosal from vaccines (AAP, 1999; AAP/USPHS, 1999).

Data on mercury levels in infants undergoing vaccination are scarce. A study on mercury disposition in 12 infants before and after hepatitis B vaccine administration (Stajich *et al*., 2000) demonstrated increased mercury blood levels, especially in pre-term infants. However, this report did not provide a sufficient basis for exposure assessment due to the limited number of subjects and collection times of samples after vaccination. Ball *et al*. (2001) calculated that cumulative mercury exposure of infants up to age 6 months who undergo multiple

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Association Between Influenza Infection and Vaccination During Pregnancy and Risk of Autism Spectrum Disorder

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IMPORTANCE Maternal infections and fever during pregnancy are associated with increased risk for autism spectrum disorders (ASDs). To our knowledge, no study has investigated the association between influenza vaccination during pregnancy and ASD.

OBJECTIVE To investigate the association between influenza infection and vaccination during pregnancy and ASD risk.

DESIGN, SETTING, AND PARTICIPANTS This cohort study included 196 929 children born at Kaiser Permanente Northern California from January 1, 2000 to December 31, 2010, at a gestational age of at least 24 weeks.

EXPOSURES Data on maternal influenza infection and vaccination from conception date to delivery date, obtained from Kaiser Permanente Northern California inpatient and outpatient databases. Influenza infection was defined by the *International Classification of Diseases, Ninth Revision, Clinical Modification* codes or positive influenza laboratory test results.

MAIN OUTCOMES AND MEASURES Clinical diagnoses of ASDs identified by *International Classification of Diseases, Ninth Revision, Clinical Modification* codes 299.0, 299.8, or 299.9 recorded in Kaiser Permanente Northern California electronic medical records on at least 2 occasions any time from birth through June 2015.

RESULTS Within this cohort of 196 929 children, influenza was diagnosed in 1400 (0.7%) mothers and 45 231 (23%) received an influenza vaccination during pregnancy. The mean (SD) ages of vaccinated and unvaccinated women were 31.6 (5.2) and 30.4 (5.6) years, respectively. A total number of 3101 (1.6%) children were diagnosed with ASD. After adjusting for covariates, we found that maternal influenza infection (adjusted hazard ratio, 1.04; 95% CI, 0.68-1.58) or influenza vaccination (adjusted hazard ratio, 1.10; 95% CI, 1.00-1.21) anytime during pregnancy was not associated with increased ASD risk. In trimester-specific analyses, first-trimester influenza vaccination was the only period associated with increased ASD risk (adjusted hazard ratio, 1.20; 95% CI, 1.04-1.39). However, this association could be due to chance ($P = 0.1$) if Bonferroni corrected for the multiplicity of hypotheses tested (n = 8). Maternal influenza vaccination in the second or third trimester was not associated with increased ASD risk.

CONCLUSIONS AND RELEVANCE There was no association between maternal influenza infection anytime during pregnancy and increased ASD risk. There was a suggestion of increased ASD risk among children whose mothers received an influenza vaccination in their first trimester, but the association was not statistically significant after adjusting for multiple comparisons, indicating that the finding could be due to chance. These findings do not call for changes in vaccine policy or practice, but do suggest the need for additional studies on maternal influenza vaccination and autism.

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Review Article

Environmental mercury contamination in China: Sources and impacts ☆

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Abstract

This review article focused on the current status of mercury (Hg) contamination in different ecological compartments in China, and their possible environmental and health impacts, focusing on some major cities. Mercury emission from non-ferrous metals smelting (especially zinc smelting), coal combustion and miscellaneous activities (of which battery and fluorescent lamp production and cement production are the largest), contributed about 45%, 38% and 17%, respectively, to the total Hg emission based on the data of 1999. Mercury contamination is widespread in different ecological compartments such as atmosphere, soil and water. There is evidence showing bioaccumulation and biomagnification of Hg in aquatic food chains, with higher concentrations detected in carnivorous fish. In terms of human exposure to Hg, fish consumption is the major exposure pathway for residents living in coastal cities such as Hong Kong, but inhalation may be another major source, affecting human health in areas with severe atmospheric Hg, such as Guiyang City (Guizhou Province). The first case study indicated that after closure of the acetic acid plant 20 years at Songyuan City (Jilin Province), 16.7% of residents' hair still contained Hg concentration in excess of 1 mg/kg (the reference dosage value, RfD set by USEPA). The second case study indicated that the male residents of Hong Kong who consumed more than four or more meals of fish per week tended to contain higher Hg in their hair, which was linked to their subfertility. There is also increasing evidence showing that skin disorders and autism in Hong Kong children are related to their high Hg body loadings (hair, blood and urine), through prenatal methyl Hg exposure. There seems to be an urgent need to identify the sources of Hg, speciation and concentrations in different ecological compartments, which may lead to high body loadings in human beings. Adverse health effects of residents living in places with a higher background level of Hg, due to long-term exposure to chronic levels of Hg through oral intake should not be overlooked.

Keywords: Mercury; China; Sources; Ecological compartment; Exposure; Health

Contents

☆ "Capsule": mercury contamination in different ecological compartments in China has potential adverse health effect to populations with high fish consumption and people living near power plants.

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RESEARCH ARTICLE

Developmental exposure to mercury chloride does not impair social behavior of C57BL/6 × BTBR F_1 mice

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Abstract

The effects of mercury (Hg) on social behavior and the mechanisms involved remain unknown. This study shows that Hg chloride ($HgCl_2$) exposure during fetal development does not impair social behavior of a mouse strain susceptible to environment-induced autistic-like behavior based on the parental phenotype. On the contrary, Hg exposure elevated the sociability of females. Since B6 mice are behaviorally normal and BTBR mice display low levels of sociability, the F_1 offspring ($B6BF_1$) of female B6 mice and male BTBR mice were used to investigate their social behavior and the effects of Hg. Developmental Hg-treatment increased the serum IgG levels of the post-natal day (pnd) 21 offspring, but not pnd70 offspring or the B6 dams. After Hg treatment, there were negligible levels of serum IgG anti-brain antibodies (Ab) in the pnd21 and pnd70 offspring as well as their dams. However, Hg did elevate IgG deposition in multiple assayed brain regions of the pnd21 offspring, but the higher levels were no longer present at pnd70. Cytokine levels were not changed in pnd21 or pnd70 brain by Hg exposure, suggesting neuroinflammation was not induced. Social behavior was assayed at pnd70. Surprisingly, Hg-treatment significantly enhanced sociability of female $B6BF_1$ offspring, but not that of the male offspring. Our data indicates that developmental exposure to $HgCl_2$ did not impair social behavior of $B6BF_1$ offspring, but it enhanced the sociability of females, which was significantly lower in adult $B6BF_1$ females than $B6BF_1$ males in the absence of any Hg exposure.

Keywords: Mercury, mouse social behavior, IgG anti-brain antibodies

Introduction

Behavioral assessment is currently the main means to diagnose autism spectrum disorders (ASD). ASD have variant degrees of social interaction and communication deficits and repetitive behaviors (American Psychiatric Association, DSM-IV-TR, 2000). The prevalence of ASD has been increasing especially in developed countries; in the US, the incidence is ~ 1 of 110 children with a ratio of 4~5 males to 1 female (Gurney et al., 2003; Mulvihill et al., 2009; Giarelli et al., 2010).

Many studies have been conducted to investigate the etiology of ASD. Genes are thought to play a substantial role in ASD pathogenesis (Kumar and Christian, 2009; Grafodatskaya et al., 2010). For instance, genes such as *MET*, *PLAUR*, and *Shank3* have been linked to abnormalities in social behavior (Eagleson et al., 2011; Peca et al., 2011). Although genetics clearly influence ASD susceptibility, in that ASD has one of the highest concordances of genetics and disorders, a recent study of twins has suggested that environment has a greater affect on ASD prevalence than genetics (Hallmayer et al., 2011). Additionally, immune dysfunction has been suggested to contribute to ASD (Ashwood et al., 2006; Goines and Van de Water, 2010). Anti-brain antibodies (Ab) have been detected in ASD children as well as their mothers (Zimmerman et al., 2007; Braunschweig et al., 2008; Singer et al., 2008; Wills et al., 2009; Goines et al., 2011). Elevated plasma inflammatory cytokines and chemokines such as interleukin (IL)-1β, IL-6, IL-12, IL-8, and interferon (IFN)-γ have been observed in ASD patients (Singh, 1996; Ashwood et al., 2011). Serum or plasma IgG has been reported as increased in ASD patients (Croonenberghs et al., 2002; Enstrom et al., 2009); however, decreased plasma IgG of ASD patients

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Low molecular weight thiols reduce thimerosal neurotoxicity in vitro: Modulation by proteins

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ABSTRACT

Thimerosal (TH), an ethylmercury complex of thiosalicylic acid has been used as preservative in vaccines. In vitro neurotoxicity of TH at high nM concentrations has been reported. Although a number of toxicological experiments demonstrated high affinity of mercury to thiol groups of the extracellular amino acids and proteins that may decrease concentration of free TH in the organism, less is known about the role of interactions between proteins and amino acids in protection against TH neurotoxicity. In the present study we examined whether the presence of serum proteins and of L-cysteine (Cys), D,L-homocysteine (Hcy), N-acetyl cysteine (NAC), L-methionine (Met) and glutathione (GSH) in the incubation medium affects the TH-induced changes in the viability, the intracellular levels of calcium and zinc and mitochondrial membrane potential in primary cultures of rat cerebellar granule cells. The cells were exposed to 500 nM TH for 48 h or to 15–25 μM TH for 10 min. Our results demonstrated a decrease in the cells viability evoked by TH, which could be prevented partially by serum proteins, albumin or in a dose-dependent manner by 60, 120 or 600 μM Cys, Hcy, NAC and GSH, but not by Met. This neuroprotection was less pronounced in the presence of proteins. Incubation of neurons with TH also induced the rise in the intracellular calcium and zinc concentration and decrease in mitochondrial membrane potential, and these effects were abolished by all the sulfur containing compounds studied and administered at 600 μM concentration, except Met. The loss of the ethylmercury moiety from TH as a result of interaction with thiols studied was monitored by ^{1}H NMR spectroscopy. This extracellular process may be responsible for the neuroprotection seen in the cerebellar cell cultures, but also provides a molecular pathway for redistribution of TH-derived toxic ethylmercury in the organism. In conclusion, these results confirmed that proteins and sulfur-containing amino acids applied separately reduce TH neurotoxicity, while their combination modulates in more complex way neuronal survival in the presence of TH.

1. Introduction

Sodium ethylmercurithiosalicylate, commonly known as thimerosal (TH) has been widely used as preservative in vaccines and topical liquid medicines. The presence of TH in vaccines was proposed to be one of factors responsible for autism in children (Bernard et al., 2001; Geier and Geier, 2004), but this concept has not been widely accepted (Madsen et al., 2003; Stehr-Green et al., 2003). However, the autistic children have significantly lower steady-state plasma levels of Met, Hcy, Cys and total glutathione than the control group (James et al., 2004; Geier and Geier, 2006). Beginning from 2001 TH has been phased out from the most of pediatric vaccines in resources-reach countries, but it is still in use in developing societies (Bigham and Copes, 2005). Other vaccines, including those against influenza, or immunoglobulin anti-D vaccine still contain TH to a maximal concentration of 25 μg Hg/0.5 ml, corresponding to 250 μM (Stratton et al., 2001). They have been administered to the wide population, including pregnant women (James et al., 2005; Geier and Geier, 2007).

Tan and Parkin (2000) using primary cultures of cerebellar granule cells (CGC) demonstrated TH neurotoxicity. They found that TH decomposes into ethylmercuric ion ($EtHg^+$) and thiosalicylic acid (TSA) and confirmed neurotoxicity of the former compound. Mercuric ions (Hg^{2+}) and their alkyl derivatives including ethylmercury are toxic to living organisms because of their strong affinity to protein cysteine thiols (Divine et al., 1999). Low molecular weight thiols, primarily L-cysteine (Cys) and reduced glutathione (GSH) modulate TH toxicity. On the one hand they are important factors in the transport and distribution of mercury throughout the body by means of molecular mimicry (Bridges and Zalups, 2005;

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Select putative neurodevelopmental toxins modify SNAP-25 expression in primary cultures of rat cerebellar granule cells

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ABSTRACT

A presynaptic protein SNAP-25 belonging to SNARE complex which is instrumental in intracellular vesicular trafficking and exocytosis, has been implicated in hyperactivity and cognitive abilities in some neuropsychiatric disorders. The unclear etiology of the behavior disrupting neurodevelopmental disabilities in addition to genetic causes most likely involves environmental factors. The aim of this *in vitro* study was to test if various suspected developmental neurotoxins can alter SNAP-25 mRNA and protein expression in neurons. Real-time PCR and Western blotting analyses were used to assess SNAP-25 mRNA and protein levels in primary cultures of rat cerebellar granule cells (CGCs). The test substances: tetrabromobisphenol-A (TBBPA), thimerosal (TH), silver nanoparticles (NAg), valproic acid (VPA) and thalidomide (THAL), were administered to CGC cultures at subtoxic concentrations for 24 h. The results demonstrated that SNAP-25 mRNA levels were increased by 49 and 66% by TBBPA and THAL, respectively, whereas VPA and NAg reduced these levels to 48 and 64% of the control, respectively. The SNAP-25 protein content in CGCs was increased by 79% by TBBPA, 25% by THAL and 21% by NAg; VPA and TH reduced these levels to 73 and 69% of the control, respectively. The variety of changes in SNAP-25 expression on mRNA and protein level suggests the diversity of the mechanism of action of the test substances. This initial study provided no data on concentration-effect relations and on functional changes in CGCs. However it is the first to demonstrate the effect of different compounds that are suspected of causing neurodevelopmental disabilities on SNAP-25 expression. These results suggest that this protein may be a common target for not only inherited but also environmental modifications linked to behavioral deficits in neurodevelopmental disabilities.

1. Introduction

The synaptosomal-associated protein 25 (SNAP-25) belongs to the evolutionarily conserved SNARE protein complex present in presynaptic and vesicular membranes; this complex is responsible for the intracellular vesicular trafficking and the regulation of exocytosis in eukaryotic cells (Jena, 2011; Cupertino et al., 2016). In neurons SNARE proteins are involved in the release of neurotransmitters and growth of plasma membrane. SNAP-25 is a multifunctional protein. This protein is required for sprouting and elongation of neurons (Osen-Sand et al., 1993; Kimura et al., 2003), plays a role in synaptogenesis (Oyler et al., 1991), is also instrumental in the recycling of the membranous proteins (Peng et al., 2013). In concert with other SNARE proteins SNAP-25 contributes to neurotransmitter release (Rizo and Xu, 2015), and modulates the activities of voltage-gated calcium channels (Matteoli et al., 2009). Its key role in cognitive functions (Gosso et al., 2006; Hou et al., 2006) and regulation of locomotor activity *in vivo* has been recognized (Hess et al., 1996).

It has been suggested, that the SNARE complex may play an important and diversified role in developmental disabilities (Cupertino et al., 2016), and altered expression of SNAP-25 may produce abnormal behavioral phenotypes in schizophrenia (Mukaetova-Ladinska et al., 2002; Thompson et al., 2003; Gray et al., 2010), attention deficit hyperactivity disorder (ADHD) (Guerini et al., 2011; Zhang et al., 2011) and autism-spectrum disorder (ASD) (Ghezzo et al., 2009). Although human genetic studies found no association of SNAP-25 with ASD, associations with behavioral deficits such as hyperactivity and cognitive function has been detected (Gosso et al., 2006; Guerini et al., 2011; Braida et al., 2015). Experiments using transgenic mouse models also demonstrated that mutations in SNAP-25 result in

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ABSTRACT

A presynaptic protein SNAP-25 belonging to SNARE complex which is instrumental in intracellular vesicular trafficking and exocytosis, has been implicated in hyperactivity and cognitive abilities in some neuropsychiatric disorders. The unclear etiology of the behavior disrupting neurodevelopmental disabilities in addition to genetic causes most likely involves environmental factors. The aim of this *in vitro* study was to test if various suspected developmental neurotoxins can alter SNAP-25 mRNA and protein expression in neurons. Real-time PCR and Western blotting analyses were used to assess SNAP-25 mRNA and protein levels in primary cultures of rat cerebellar granule cells (CGCs). The test substances: tetrabromobisphenol-A (TBBPA), thimerosal (TH), silver nanoparticles (NAg), valproic acid (VPA) and thalidomide (THAL), were administered to CGC cultures at subtoxic concentrations for 24 h. The results demonstrated that SNAP-25 mRNA levels were increased by 49 and 66% by TBBPA and THAL, respectively, whereas VPA and NAg reduced these levels to 48 and 64% of the control, respectively. The SNAP-25 protein content in CGCs was increased by 79% by TBBPA, 25% by THAL and 21% by NAg; VPA and TH reduced these levels to 73 and 69% of the control, respectively. The variety of changes in SNAP-25 expression on mRNA and protein level suggests the diversity of the mechanism of action of the test substances. This initial study provided no data on concentration-effect relations and on functional changes in CGCs. However it is the first to demonstrate the effect of different compounds that are suspected of causing neurodevelopmental disabilities on SNAP-25 expression. These results suggest that this protein may be a common target for not only inherited but also environmental modifications linked to behavioral deficits in neurodevelopmental disabilities.

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