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Neuroimmune Responses to Toxic Agents: Comparison of Organometal Electrophiles Using Detection of Antibodies to Neural Cytoskeleton and Myelin as Biomarkers

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Abstract: Chemicals, including heavy metals, are of health and ecological concern due to environment release from industrial and agricultural activity. Neurotoxicity is of particular concern because of effects on the developing Nervous System (NS) and contribution to neurodegeneration in later life. A pressing need exists to develop and validate biomarkers of neurotoxicity to monitor those at risk and implement successful intervention strategies. Fischer 344 rats were given one of three documented neurotoxic organometals: trimethyltin (TMT), methylmercury (MeHg) or trimethyl lead (TML) at 16 ppm in the drinking water and compared to water-only controls (n = 8/group). Detection of serum antibodies, IgM and IgG isotypes, against neurofilaments (NF), astrocytic Glial Fibrillary Acidic Protein (GFAP) and Myelin Basic Proteins (MBP) were used as biomarkers of neurotoxicity confirmed by measurement of brain GFAP, a marker of astrogliosis, in rats on day 12 of exposure. While sera from control rats did not have detectable levels of antibodies against neural proteins, sera from rats exposed to all three metals had antibodies, both IgM and IgG, against all neural antigens, with the exception of IgM against MBP which was not detected in sera of MeHg-exposed rats. Serum IgM titers against NF-L and MBP were significantly ($p \leq 0.001$) higher with TML exposure. Serum IgG titers against NF and GFAP were more prevalent and significantly ($p \leq 0.001$) higher in TMT-exposed rats, compared to the other two organometals. This suggests that neurotoxicity was more advanced with TMT, an observation substantiated by greater generalized toxicity indicated by reduced body weight and hyperexcitability after the first week of exposure. Furthermore, anti-GFAP, IgM and IgG, were consistently higher in this group, the only metal of the three, reported to be gliotoxic. Brain GFAP was significantly ($p \leq 0.001$) elevated in hippocampus of rats exposed to TMT or TML and in the cerebellum for those exposed to MeHg. This regional vulnerability of the brain is consistent with the neurotoxicity of these agents. Despite treatment of rats with equivalent levels of oragnaometals in the drinking water and similarities as electrophiles that complex with nucleophilic molecules, other mechanisms underlie their differential neurotoxicity. Proposed mechanism of neurotoxicity and immune activation are reviewed and discussed. This study further supports the utility of neuroantibody detection as a biomarker of neurotoxicity.

Key words: Biomarkers, neurotoxicity, autoantibodies, GFAP, neurofilaments, myelin, metals

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INTRODUCTION

Heavy metals continue to pose a health and environmental risk due to their discharge into the environment from industrial and agricultural sources. Neurotoxicity contributing to impaired cognitive function in children and neurodegenerative diseases in later life are major concerns associated with exposure to these and other agents (Lidsky and Schneider, 2006; Landrigan *et al*., 2005; Caban-Holt *et al*., 2005). Organic metals, in particular, pose a risk to the nervous system because of their lipophilicity which allows them to readily gain access to the brain and because as electrophiles they are capable of inducing oxidative stress to which the nervous system is particularly vulnerable (Aldridge *et al*., 1977; Chang, 1990; Verity, 1990; Ali *et al*., 1992; Dreiem *et al*., 2005). As electrophiles, metals target nucleophilic molecules such as amino acids, peptides and the cytoskeleton (Berthon, 1995; Graff and Reuhl, 1996; Farakas and Sóvágá, 2002). Several organic metals, including those studied here interact with amino acids, particularly those containing sulfhydryl groups (Fig. 1) with relatively strong formation constants (Table 1). MeHg (Fig. 1A) for example, complexes with thiol-containing ligands. Amino acids containing thiol groups such as cysteine (Fig. 1A2), homocysteine, the thioether group of methionine (Fig. 1A3), the antioxidant peptide glutathione (Fig. 1A4), metallothionein and proteins such as albumin react with MeHg (Chang, 1990; Verity and Sarafian, 2000). The amino acids glycine (Fig. 1A5) and β-alanine complex with MeHg through nitrogen and oxygen atoms, respectively. The MeHg-cysteine and MeHg-glutathione complexes have high formation constants (Table 1). However, studies have shown that these ligands are very labile and can easily be exchanged, a mechanism believed to facilitate MeHg entry into the brain. MeHg easily forms a complex with l-cysteine in blood plasma which is structurally similar to the amino acid methionine and enters the blood brain barrier via the methionine carrier (Chang, 1996). There, the cysteine exchanges with glutathione in a reaction catalyzed by γ-glutamyl transpeptidase (Verity and Sarafian, 2000). TMT (Fig. 1B1), forms penta and hexa coordinate complexes with ligands (Fig. 1B2).

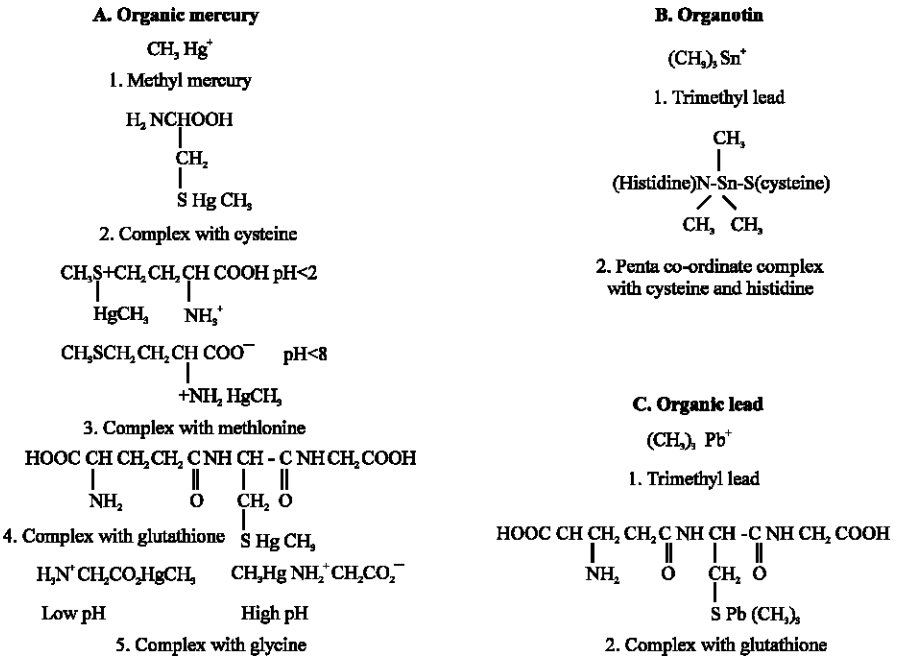

Fig. 1: Interactions of electrophiles MeHg (A), TMT (B) and TML (C) with amino acids or glutathione that may underlie their transport or toxicity

Table 1: Formation constants, K_f (log β) for organometals and amino acids and glutathione*

Metal cation	Amino acid	log β
CH_3Hg^+	Cysteine	15.70
	Methionine	7.40
	Glycine	7.88
	Glutathione	15.90
$(CH_3)_3Sn^+$	Cysteine	10.54
	Methionine	7.73
$(CH_3)_2Pb^+$	Cysteine	5.97

*: Compiled from Perkins (1952); Hynes and O'Dowd (1987); Berthon (1995); Farakas and Sóvágá (2002)

This has been extensively studied for TMT and rat hemoglobin, for which it has high affinity (Krinke, 2000a). In addition, the specific organic molecule associated with the alkyltin determines its toxicity. Methyl groups, as compared to ethyl groups, make TMT preferentially neuronotoxic, whereas triethyltin is myelinotoxic (Krinke, 2000a, b). TML, like MeHg and TMT, is also a cation (Fig. 1C1). Similar to MeHg, TML complexes predominantly with sulfur containing amino acids like cysteine and peptides like glutathione (Fig. 1C2). Although to date, no single target or mechanism has been defined as underlying metal-induced neurotoxicity, their fundamental interaction with biological nucleophilic molecules is undoubtedly involved. Indeed, LoPachin and Barber (2006) have recently proposed that the interaction of electrophilic neurotoxicants, including metals, with reactive cysteine residues through their thiolate anions may explain the preferential targeting of particular neuronal structures such as synaptic proteins. These residues are part of catalytic triads that regulate enzymes, membrane transporters and ion channels. In addition to the cytoskeleton, these structures and processes are targeted by electrophilic metals (Mailman *et al*., 1996; Narahashi, 1996; Graff and Reuhl, 1996).

Regardless of the mechanism(s) by which pharmacological or environmental agents, including metals, precipitate neurotoxic injury, it is equally important to identify nervous system insult as early as possible in order to design and implement suitable intervention strategies, particularly when damage may be minimal and even reversible. The development of biomarkers of effect is particularly relevant with the growing interest and concern about the contribution of environmental chemicals to neurodegenerative disorders such as Alzheimer's (AD), Parkinson's (PD) and Amyotrophic Lateral Sclerosis (ALS) (Oteiza *et al*., 2004; Landrigan *et al*., 2005; Caban-Holt *et al*., 2005). It becomes even a greater imperative when we consider the potential for detrimental, often irreversible, impact on the developing nervous system (Grandjean and Landrigan, 2006). The development of sensitive biomarkers of neurotoxic effect and/or susceptibility is an area in which neurotoxicology has lagged behind other toxicology disciplines because of inaccessibility and heterogeneity of nervous system targets (Gil and Pla, 2001). Based on the literature and preliminary studies, we have proposed that detection of autoantibodies against neurotypic and gliotypic antigens be a surrogate to document insult to the nervous system (El-Fawal, 1996; El-Fawal *et al*., 1999). This approach is not without precedent. Serum autoantibodies to nerve growth factor, Glial Fibrillary Acidic Protein (GFAP) and the astrocytic protein S100b have been reported in several mental and neurological disorders, including PD (Terryberry *et al*., 1998; Poletaev *et al*., 2000; Orr *et al*., 2005). In ALS patients, antibodies against Ca^{2+} voltage-operated channels and antineuronal proteins, including neurofilament-L (NF-L), have been found in sera and cerebrospinal fluid (CSF) (Rowland and Shneider, 2001; Nieborj-Dobosz *et al*., 2006). In AD patients, there have been reports of antibodies to GFAP, Aβ peptide and other neural proteins in CSF and serum (Frank *et al*., 2003; Bouras *et al*., 2005). Chronic spinal cord injury patients have serum antibodies to GM1 ganglioside and Myelin-Associated Glycoprotein (MAG) (Hayes *et al*., 2002). These studies demonstrate that autoantibody detection is indicative of nervous system insult. Although these neurodegenerative conditions are not recognized as autoimmune disorders, the presence of autoimmunity does indicate injury to neural tissue and, therefore, in the context of neurotoxicology would provide a useful biomarker to assess and monitor potential neurotoxicity (El-Fawal *et al*., 1999).

The present study was undertaken in a continued effort to evaluate the detection of autoantibodies against neurotypic and gliotypic proteins in blood serum as a peripheral marker of neurotoxicity following exposure to three organometals: TMT, MeHg and TML. The choice of TMT was based on its successful use as a denervation tool for evaluating GFAP immunochemistry and immunoassay as markers of neurotoxicity in animal studies and post-mortem brain (O'Callaghan *et al*., 1999). MeHg and TML were chosen based on previous studies documenting their neurotoxicity in our laboratory using GFAP measurement as a marker of neurotoxicity and preliminary evaluation of neuroantibody detection (Gong *et al*., 1995; El-Fawal *et al*., 1996).

MATERIALS AND METHODS

Animals and Treatments

Male Fischer 344 rats (six weeks of age; Taconic Farms, Germantown, NY) were divided into 4 groups (n = 8/group): control, TMT-, MeHg-and TML-treated. Rats were housed singly in plastic tub cages with shred bedding in a temperature- (22±2°C) and humidity-controlled (50±10%) colony room maintained on a 12 h light/12 h dark schedule. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of New York University Medical Center (New York, NY). Rats were exposed to equivalent doses of 16 ppm of TMT (hydroxide), MeHg (chloride) or TML (chloride). TMT was obtained from K and K Laboratories (Division of ICN Biomedical, Cleveland, OH). MeHg and TML were obtained from Alfa Products (Danvers, MA). Water consumption and body weight were recorded at regular intervals to estimate actual dose (Fig. 2). Organometal-treated rats and distilled water controls (for each time point) were sacrificed by decapitation at 12 days of treatment. At these time points serum was collected post-decapitation using a ventricular puncture. A 2 mL blood sample was collected and placed in a coagulation tube for serum separation. Following centrifugation, serum was aspirated into 0.5 mL polyethylene eppendorf tubes and frozen at -85°C for later assay of autoantibodies. Simultaneous to blood collection, brains were removed from the skull, the frontal cortex, hippocampus and cerebellum were immediately dissected and the tissue then was homogenized by sonification in hot (85-95°C) 1% SDS for subsequent GFAP immunoassay.

Brain GFAP Determination

Brain region GFAP levels were determined using the sandwich enzyme-linked immunosorbent assay (ELISA) developed by O'Callaghan (1991, 2002). Briefly, total protein of each brain region was determined colormetrically with an assay kit (BCA, Pierce, Rockford, Illinois). Bovine serum albumin was used as a standard. Samples were normalized for total protein prior to performance of the ELISA. A GFAP standard (25 μg-1000 μg protein/μg homogenate) was generated using a stock of control hippocampus homogenate. Flat-bottomed Immulon microtiter plates (Fisher Scientific, NJ) were coated with 1.0 μg/100 μg/well polyclonal anti-GFAP (Dako, Carpenteria, California) for 1 h at 37°C. Microtiter plates were washed with phosphate buffered saline (PBS, pH 7.4), blocked for 1 h with Blotto (5% nonfat dry milk in PBS) then 100 μL of standard or normalized sample were added for 1 h. After washing with PBS-0.5% Triton X-100, plates were incubated for 1 h with monoclonal anti-GFAP (Oncogene Research Products, Boston, MA), washed with PBS-0.5% Triton X-100, then alkaline phospatase-conjugated anti-rat antibody (Jackson Immunoresearch, West Grover, PA) was added for 30 min. Plates were washed with PBS-0.5% Triton X-100. The alkaline phosphatase substrate, p-nitrophenylphosphate (Bio-Rad, Richmond, CA), was then added. The reaction was stopped after 30 min by adding 100 μL of 0.4 N NaOH. Absorbance was read at 405 nm in Spectramax Plus microtiter plate reader (Tecan Spectrafluor Plus).

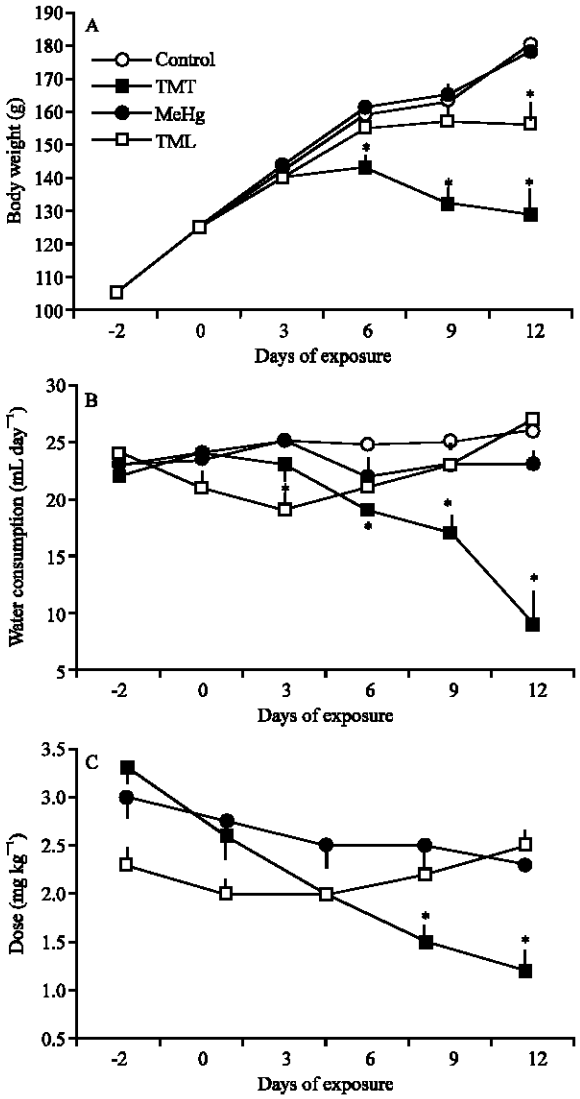

Fig. 2: Growth curves (A), water consumption (B) and estimated dose (C) of TMT, MeHg and TML in rats administered 16 ppm of organometal in the drinking water. Metal treated rats were compared to control rats receiving distilled water only. There was a significant ($*p \leq 0.05$) decrease in body weight and water consumption in rats treated with TMT at the end of the first week of exposure. This was paralleled by a significant ($*p \leq 0.05$) decline in estimated dose for this group. Significant decreases in body weight were measured only on day 12 for TML-treated rats. Each point represents the mean±SEM

Serum Autoantibody Determination

Serum levels of autoantibodies to neurotypic and gliotypic proteins were determined as described by El-Fawal *et al*. (1996). Purified spinal cord proteins (NF-L; NF-M; NF-H or GFAP; American Research Products, Belmont, MA) or MBP (Sigma-Aldrich, St. Louis, MO) were prepared in a 10 mM

Tris-buffered saline (pH 7.4) at a concentration of 5 μg mL^{-1}, except for MBP, which was used at a concentration of 25 μg mL^{-1}. Flat-bottomed Immulon microtiter plates (Fisher Scientific, NJ) were coated with 100 μL of the protein solution and incubated overnight at 4°C. The plates were washed three times with 10 mM Tris containing 0.05% Tween-80. Non-specific binding sites were blocked for 30 min at room temperature with 0.5% skim milk solution prepared in the Tris Buffer. Serum dilutions of 1:100 were prepared in the skim milk solution and 100 μL added per well, in triplicate. Plates were incubated for 2 h at room temperature then washed 3 times with skim milk solution containing 0.05% Tween-80. Alkaline phosphate goat anti-rat IgG or IgM (Jackson Immunoresearch, West Grover, PA) at a concentration of 1:3000 was added to each well and the plates allowed to incubate for 1 h. Following three washes with skim milk-Tween-80 solution and 2 washes with 10 mM Tris, the alkaline phosphatase substrate (p-nitrophenyl-phosphate, Bio-Rad, Richmond, CA) was added. The reaction was stopped by the addition of 100 μL 0.4 N NaOH per well. Plates were read at 405 nm in a plate reader (Tecan Spectrafluor Plus). Generation of standard curves for calculation of ng mL^{-1}. equivalence (ng mL^{-1} eq) was performed using commercially available rabbit polyclonal antibodies against NF, GFAP (Encor Biotechnology, Gainesville, FL) or MBP (Biomeda, Inc., CA). The range of the 8 point standard curve was 0 to 2,000 ng mL^{-1} and the threshold of detection for the assay was 0.20 ng mL^{-1}.

Statistical Analysis

Statistical comparisons of GFAP levels and IgM and IgG titers were by ANOVA and Newman-Keuls test for multiple comparisons using Statistics Program for the Social Sciences (SPSS) software (Chicago, IL).

RESULTS AND DISCUSSION

Exposure to 16 ppm TMT caused significant weight loss in rats as early as 6 days of exposure and continued for the duration of the study (Fig. 2A). This was paralleled by a significant decrease in water consumption (Fig. 2B) and the estimated dose received (Fig. 2C). TMT-exposed rats were irritable and displayed hyperexcitable and aggressive behavior. In contrast, there was no statistically significant change in body weight for rats exposed to 16 ppm of MeHg, although dose showed a declining trend (Fig. 2). Statistically significant declines in body weight of rats exposed to 16 ppm TML was only evident on day 12 of the study (Fig. 2A). Although there was an initial decrease in water consumption, significant only at day 3 of exposure, this increased at the end of the study (Fig. 2B) and was mirrored by the estimated dose (Fig. 2C).

There were no detectable autoantibodies to any of the five nervous system antigens in rats receiving water only. Autoantibodies, IgM and IgG isotypes, to NF triplet proteins were detected at 12 days of exposure to 16 ppm of organometals in the drinking water. Titers of anti-NF-L being the most predominant (Fig. 3A). IgM levels to NF-L and MBP were highest in TML-exposed rats, compared to titers of TMT-or MeHg-exposed animals (Fig. 3A). In fact, MeHg-exposed animals did not have detectable levels of anti-MBP IgM. In contrast, IgM against GFAP, the astrocytic cytoskeletal protein, was greatest in sera of rats exposed to TMT (Fig. 3A). In these same rats, IgG levels against NF-L, NF-M, NF-H and GFAP significantly higher compared to levels in sera of MeHg-or TML-exposed animals, while anti-MBP IgG was higher for these two latter groups (Fig. 3B).

Hippocampal GFAP levels were significantly higher than control in TMT-or TML-treated rats (Fig. 4). For the MeHg-treated rats, the greatest change was in the levels of cerebellar GFAP (Fig. 4). Levels of GFAP in the frontal cortex of animals treated with any of the organometals were comparable to controls at this time point (Fig. 4).

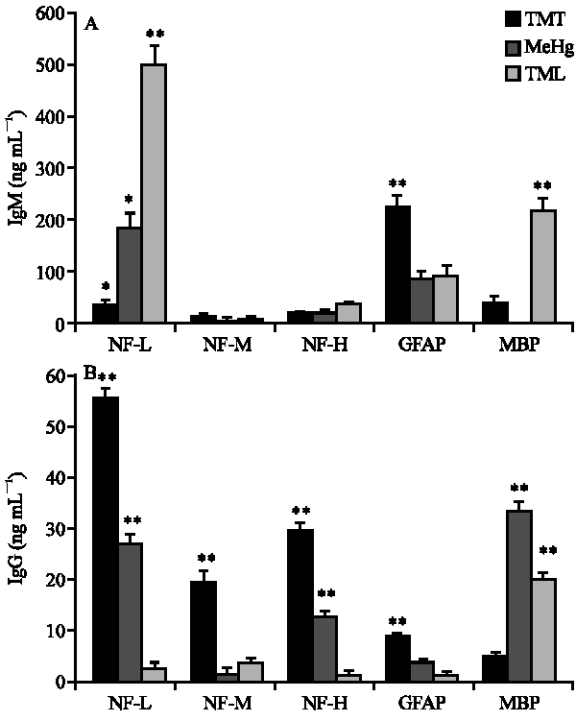

Fig. 3: Autoantibody, IgM (A) and IgG (B) titers determined by direct ELISA against neurotypic: NF-L, NF-M and NF-H; gliotypic: GFAP and MBP antigens in sera of rats exposed to 16 ppm TMT, MeHg or TML at 12 days of exposure in the drinking water (n = 8/group). There were no detectable IgM or IgG against these proteins in sera of control rats. In sera of TML-exposed rats (A), IgM against NF-L and MBP were more prevalent and significantly (**$p \leq 0.001$) higher compared to IgM titers in sera of TMT-or MeHg-exposed rats. In fact, MeHg-exposed rats did not have detectable IgM against MBP, however, anti-NF-L, IgM titers, for this group, were also significantly (*$p \leq 0.001$) higher than those in TMT-exposed rats. This latter group, had the highest (**$p \leq 0.001$) anti-GFAP, IgM titers, compared to MeHg or TML-exposed rats. This group also had the highest (**$p \leq 0.001$) serum IgG titers against the three NF proteins and against GFAP (B), but not MBP. The highest (**$p \leq 0.001$) IgG titers against MBP were in the sera of rats exposed to MeHg. A greater prevalence of IgG in TMT-exposed rats suggests the more advanced neurotoxicity in this group. Ranking of neurotoxicity based on IgG levels against the cytoskeletal proteins suggests the following order: TMT >MeHg>TML. Bar height indicates the mean titer±SEM

This study demonstrated that autoantibodies to neurotypic and gliotypic proteins could be used to detect neurotoxicity and neural damage. Here we used the denervation tool TMT, which had been previously used to validate and establish GFAP as biomarker of neurotoxicity (O'Callaghan, 1988) and MeHg, previously used to test the utility of neuroantibody detection (El-Fawal *et al*., 1996). Both TMT and TML are reported to preferentially target the pyramidal neurons of the hippocampus, producing seizure activity and cell death (Chang, 1996; Spencer, 2000; Krinke, 2000b). Hippocampal injury and neurodegeneration is substantiated in the present study by the occurrence of astrogliosis measured by immunoassay (Fig. 4). The increase in GFAP with TML exposure at this level is consistent with earlier reports by Gong *et al*. (1995). In contrast, MeHg, may target the hippocampus,

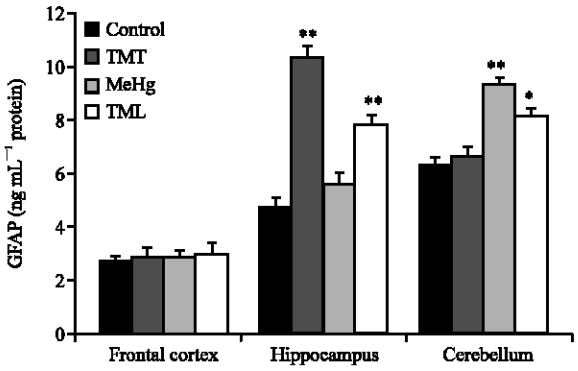

Fig. 4: GFAP levels determined by sandwich ELISA in brains of control rats and in rats exposed to TMT, MeHg or TML (n = 8/group). Hippocampal GFAP levels were significantly (**$p \leq 0.001$) higher in TMT-and TML-exposed rats compared to hippocampi of control or MeHg-exposed rats. In contrast, cerebellar GFAP levels were significantly higher in MeHg-(**$p \leq 0.001$) and TML-exposed (*$p \leq 0.05$) rats compared to cerebella of control or TMT-exposed rats. An increase in GFAP, a marker of astrogliosis and neurotoxicity, in hippocampi of TMT-or TML-exposed rats and in the cerebellum of MeHg-exposed rats, is consistent with pattern of neurotoxicity documented for these agents. It also substantiates that neuroantibody detection is a biomarker of neurotoxic insult. Bar height indicates the mean titer±SEM

but preferentially causes injury to cerebellar granule cells and the visual cortex (Chang, 1996; Verity and Sarafian, 2000). This was confirmed in the present study by the significant increases in cerebellar GFAP levels (Fig. 4), which is consistent with an earlier study from our laboratory (El-Fawal *et al*., 1996). Furthermore, for all exposures used in the present study, neuronal damage and loss was indicated by the detection of serum IgM and IgG directed against the NF proteins not found in sera of controls (Fig. 3).

There were no detectable autoantibodies to any of the five nervous system antigens in sera of control rats receiving only water. In the context of this study to test whether autoantibodies could be used as biomarkers of neurotoxicity, this is relevant. Fischer 344 rats have been shown to be resistant to the development of experimentally-induced autoimmunity in response to exogenous antigen (Wilder *et al*., 2000). That humoral autoimmune responses were detected in Fischer 344 rats in the present study strengthens the evidence for autoimmunity resulting from endogenous antigen release as a result of organometal-induced neural damage.

The prevalence of autoantibodies to neurofilaments is consistent with the neuronopathy attributed to TMT, MeHg and TML. The predominance of IgM and IgG against NF-L may reflect the abundance of this antigen relative to the other neurofilaments. Neurofilaments, from low to high molecular weights, exist in a ratio of 4:2:1 or 6:2:1 (Scott *et al*., 1985; Poon *et al*., 2004). On the other hand, high levels of autoantibody titers to NF-H, relative to NF-M, may reflect the greater number of immunogenic epitopes associated with the larger protein.

Compared to MeHg-and TML-exposed rats, TMT-exposed rats had higher levels of IgG against all neural proteins used in the present study. Appearance of IgG indicates successful isotype switching from IgM and is the immunoglobulin associated with the development of immunological memory (Kindt *et al*., 2007). This suggests earlier neurodegeneration in TMT-exposed rats (Fig. 3B). This is further suggested by the still higher levels of IgM against NF proteins in MeHg-and TML-exposed rats compared to TMT-exposed rats (Fig. 3A).

The presence of detectable autoantibodies to MBP likely reflects myelin degeneration secondary to neuronal loss (Spencer, 2000). Earlier studies of single doses of TMT, in developing rats, indicate that TMT does not significantly alter myelin protein levels up to post-natal day 66 (Miller and O'Callaghan, 1984). While both IgM and IgG against MBP were found in sera of TMT or TML-exposed rats, interestingly, only IgG against MBP were found in rats exposed to MeHg. Although this is consistent with our earlier report (El-Fawal *et al*., 1996), outside of developmental studies, few reports indicate direct myelinotoxicity of mercurial compounds (Grundt *et al*., 1980). In contrast, Stewart *et al*. (2006), using MRI, report significant parietal white matter (myelinated fibers) loss in brains of adult humans exposed to organolead in earlier life. In these individuals, there was a significant inverse correlation with lead accumulation in the tibia.

An interesting observation was the detection of autoantibodies against GFAP. Although decreases in brain GFAP have been reported before, these decreases were likely attributable to stress-induced corticosteroid release and not gliotoxicity (O'Callaghan *et al*., 1991; Little *et al*., 1998; Reed *et al*., 2006). In the present study, autoantibodies to GFAP would suggest the likelihood of some astrocytic injury. A recent study of astrocytes in a streptozotocin-induced diabetes model indicates ischemia-induced astrocyte death with a decrease in GFAP immunostaining (Muranyi *et al*., 2006). Studies of TMT's effects on cultured astrocytes indicate that the organometal is toxic to astrocytes (Karpiak and Eyer, 1999; Karpiak *et al*., 2001; Cristofol *et al*., 2004), possibly attributable to glutamate activity (Chen *et al*., 2000; Huck *et al*., 1984; Karpiak *et al*., 2001). Alternatively, according to Brock and O'Callaghan (1987), GFAP levels eventually return to near control levels after TMT, findings consistent with the eventual decline in astrocytic hypertrophy observed for a wide variety of neurotoxic exposures and suggests turnover and release of GFAP during the course of neurotoxicity (O'Callaghan and Sriram, 2005). Regardless of the mechanism, in the context of this study, GFAP would have to have been presented as an autoantigen to stimulate autoantibody production. As noted earlier, antibodies to GFAP have been detected in other NS insults (Poletaev *et al*., 2000).

That the exposures used here and therefore antibody generation, did produce neurotoxic injury is supported by occurrence of astrogliosis as indicated by increases in brain GFAP. Astrogliosis, hypertrophy of astrocytes secondary to neuronal injury, is considered the universal cellular reaction to damage of the central nervous system (Garman *et al*., 2001; O'Callaghan and Sriram, 2005). Thus neurotoxicity was confirmed by elevations in GFAP in the hippocampus of those rats exposed to TMT or TML and in the cerebellum of those exposed to MeHg. This regional astrogliosis is consistent with the documented neurotoxicity of these agents (Chang, 1996; Spencer, 2000).

How cytoskeletal proteins may be made available as antigens to stimulate generation of autoantibodies has not been delineated, however, several interactions between metals and these proteins may facilitate this process. Waterman *et al*. (1994) working with inorganic lead reported the potential for metal-induced increased immunogenicity and generation of neoantigens. Intermediate filaments (IF), which include NF and GFAP, include a protease-sensitive amino head region, an α-helical coiled-coil rod domain of approximately 40 kD and a carboxy terminal tailpiece. The head and tailpiece are hypervariable non-α-helical sequences (Weber and Geisler, 1985). It is the rod, however, where thiol-containing cysteine and methionine residues are found (Geisler *et al*., 1983). It is feasible that metal-thiolate complexes (Fig. 1, Table 1) formation interferes with NF assembly or results in aggregation. Disruption of NF by triethyl lead (Et_3L) and tetraethyl lead (Et_4L) has been reported by Niklowitz (1974, 1975) and Zimmerman *et al*. (1987). Studying Et_3L and Et_4L, Niklowitz reported aggregation of NF to form neurofibrillary tangles in hippocampal pyramidal neurons of rabbit brain. In contrast, Zimmerman and co-workers studying the effects of Et_3L, *in vitro* on neuroblastoma cells, reported collapse and disassembly of NF. The authors speculate that this is due to weakening of hydrophobic bonds or formation of complexes with cysteine residues of the core. It is possible that metals may also interfere with NF phosphorylation status. In cerebral cortex slices of rats, MeHg has

been shown to reduce phosphorylation of NF-L, NF-M and GFAP (Moretto *et al*., 2005) and induce hyperphosphorylation of NF-H (Funchal *et al*., 2006). While phosphorylation of NF is necessary for assembly and axonal transport of the cytoskeleton (Jung and Shea, 1999; Ackerley *et al*., 2003), hyperphosphorylation is associated with aggregation of the cytoskeleton and neurodegeneration (Giasson and Mushynski, 1996; Ackerley *et al*., 2003; Shea *et al*., 2004; Veeranna *et al*., 2004). Contributing to cytoskeletal derangement and neuronal death is the oxidative stress and excitoxicity induced by heavy metal exposure. Lead is known to complex with glutathione (Fig. 1), inhibit glutathione peroxidase, synergize with glutamate-induced excitotoxicity, interfere with oxidative phosphorylation (Aldridge *et al*., 1977; Savolainen *et al*., 1998a, b; Oteiza *et al*., 2004) and increase intracellular Ca^{2+} by displacing it from the endoplasmic reticulum (Tamse *et al*., 1996). Similarly, contributing to MeHg neurotoxicity is the increased production of Reactive Oxygen Species (ROS) in the cerebellum and striatal synaptosomes (Ali *et al*., 1992; Atchison and Hare, 1994; Dreiem *et al*., 2005). In astrocytes, MeHg selectively interferes with the transport of cysteine and cystine, reduces glutathione content, inhibits glutamate uptake and increases it efflux, thereby increasing extracellular glutamate and excitotoxic neurodegeneration (Fonnum and Lock, 2004; Aschner *et al*., 2007). Increased hippocampal ROS production and interference with mitochondrial function has also been demonstrated for TMT exposure (Aldridge *et al*., 1977; Ali *et al*., 1992; Gunasekar *et al*., 2001). Furthermore, TMT decreases glutathione (Shin *et al*., 2005) and induces glutamate efflux from rat hippocampal preparations (Patel *et al*., 1990; Patterson *et al*., 1996). Gunasekar *et al*. (2001) have demonstrated that TMT-induced ROS production may result in apoptosis or necrosis depending on dose. Glutamate excitotoxicity due to oxidative stress is associated with increased Ca^{2+} from mitochondria stores in neurons (Horn *et al*., 2002). Increases in intracellular free Ca^{2+} activate the Ca^{2+}-activated neutral protease, calpain, which degrades NF triplet proteins (El-Fawal *et al*., 1990; El-Fawal and Ehrich, 1993; Goll *et al*., 2005). Calpain is believed to participate in several neurodegenerative disorders, including Multiple Sclerosis, AD, PD, ALS, Huntington's Disease and in glutamate-induced excitotoxicity (Goll *et al*., 2005; Shields *et al*., 1999). In neurons, glutamate has been shown to activate the transcription factor NFκB via calpain activation (Schölzke *et al*., 2003). Calpain's participation in neurodegeneration as a result of oxidative stress is inhibited by calpain inhibitors or inhibitors of ROS and nitric oxide generation (Banik *et al*., 1998; See and Loeffler, 2001; Moore *et al*., 2002). Recently, MeHg-induced degeneration of isolated cerebellar neurons has been shown to be mediated by calpain activation (Sakaue *et al*., 2005). As described above, there are several inter-related mechanisms in metal-induced neurodegeneration. The accumulation and subsequent removal of protein degradation products resulting from neurodegeneration, particularly intracellular proteins, may provide an opportunity for immune activation mediated by astrocytes and microglia.

That nervous system antigens, including those from the CNS, can stimulate adaptive immune activation detected in the periphery is suggested by the literature (Targoni *et al*., 2001; de Vos *et al*., 2002). Therefore, it seems feasible that in the presence of neuronal degeneration, demyelination and glial damage, such as that precipitated by environmental chemicals in either CNS or PNS, proteins specific to these structures are presented as autoantigens, particularly intracellular proteins like those of the cytoskeleton (i.e., NF and GFAP). Development of this adaptive immune response may occur in the CNS with the participation of microglia and astrocyte, including the active recruitment of lymphocytes. Microglia, monocytes of the brain and spinal cord, are antigen presenting cells that constitutively express Class II MHC required for development of cellular and humoral immunity, including autoimmunity (Olson and Miller, 2004; Town *et al*., 2005). When activated, these cells produce a host of cytokines, as well as nitric oxide. Their role in neurodegeneration, including AD, MS, PD, ALS and neurotoxic insult has been recently reviewed (Streit *et al*., 2004; Block and Hong, 2005). Microglial activation and cytokine production (e.g., tumor necrosis factor-α; TNF-α), by TMT treatment, have been demonstrated in cell culture (Eskes *et al*., 2003; Reali *et al*., 2005; Figiel and

Dzwonek, 2007) and may rely on astrocyte participation (Rohl and Sievers, 2005). Microglia from rats administered TMT also elaborate interleukin (IL)-16, or lymphocyte chemoattractant factor (Guo *et al*., 2004), as well as monocyte-chemoattractant protein (MCP-1) (Little *et al*., 2002), which may set the stage for developing an immune response to autoantigens in absence of overt inflammation. Charleston *et al*. (1996) reported an increase in microglia in thalami of macaque monkeys exposed to MeHg over the course of 18 months and lead significantly increases microglia number in hippocampi and cerebral cortices, but not cerebella of rats (Struzynska *et al*., 2003). This group also demonstrated astrocyte activation in the hippocampus and cortex accompanied by an increase in TNF-α, IL-1β and IL-6. Oxidative stress, where TNFα is produced, favors the development of cell death, through mitochondrial dysfunction and the exposure of autoantigens, or alternatively, drugs and environmental chemicals that cause cellular injury alter proteins and generate neoantigens that are immunogenic and therefore, precipitate autoimmune responses (Kannan, 2006). Adaptive immunity to nervous system antigens may also develop outside of the CNS in the peripheral lymph nodes and spleen. Studies of neurodegenerative autoimmune models have demonstrated that autoantigens are transferred to these peripheral tissues for presentation, particularly in the early stages of autoimmunity development (Targoni *et al*., 2001; de Vos *et al*., 2002). Whether autoantibodies are produced in the CNS or periphery, they are found in the CSF and serum. In this way the immune system may provide a means whereby cellular damage in the nervous system can be documented and measured in serum (El-Fawal, 1996; El-Fawal *et al*., 1999).

Despite the use of comparable exposures to organometals that share chemical properties and biochemical targets as electrophiles, this is evidently not sufficient to account for differential neurotoxicity. Neuroantibody profiles, based on IgG, suggest that TMT is the more toxic of three organometals used. Furthermore, while antibodies were found against all the neuroantigens tested for, titer levels and prevalence of any particular immunoglobulin differed between groups. The present study confirms that the detection of neuroantibodies, in serum, directed against neurotypic and gliotypic proteins, may be used as biomarkers of neurotoxicity and provide a means of monitoring neurotoxic effects in human. Aligning particular profiles with particular exposures is currently the subject of on-going research.

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