# Lead Alters the Immunogenicity of Two Neural Proteins: A Potential Mechanism for the Progression of Lead-induced Neurotoxicity

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Some heavy metals have been suspected of playing a role in the pathogenesis of nervous system diseases such as multiple sclerosis, amyotrophic lateral sclerosis, and Alzheimer's disease. In these disorders, autoantibodies against neural proteins are evident at some stage of the disease. Lead is known to affect both the immune and nervous systems. Work in our laboratory has shown that lead exposure leads to the production of autoantibodies against neural proteins, including myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP). We hypothesize that lead aggravates neurological disease by enhancing the immunogenicity of nervous system proteins, including MBP and GFAP. To test this hypothesis, lead-altered protein was prepared by incubating MBP or GFAP with lead acetate for 24 hr. On days 0, 14, and 28, mice received inoculations with either saline, native protein, or lead-altered protein. Anti-MBP and anti-GFAP, isotypes IgM and IgG, were measured in sera by ELISA on day 38. Sera of mice treated with lead-altered MBP had statistically higher anti-MBP IgG titers than both control and native MBP-immunized mice. An analogous response was seen in mice immunized with lead-altered GFAP. Supernatants from lectin-stimulated splenocytes were also examined for antibody titers and for interleukin 2 (IL-2) and interleukin 6 (IL-6) levels. A significant increase in IL-6 production was seen in mice immunized with lead-altered MBP but not with lead-altered GFAP. No changes were observed in the IL-2 levels of mice immunized with either lead-altered protein. These results demonstrate that lead does enhance the immunogenicity of two neural system proteins MBP and GFAP and suggests a possible mechanism for an immunologically mediated progression of lead-induced neurotoxicity. In addition, the IL-6 results suggest that lead-altered MBP may induce antibody production through a different immunological mechanism from lead-altered GFAP. *Key words:* immunogenicity, lead, neural protein, neurotoxicity. *Environ Health Perspect* 102:1052–1056 (1994)

The effects of lead on the immune system and the nervous system have been studied extensively but separately. The nervous system was believed to be an immunologically privileged site because it lacks lymphatic drainage and is shielded from the blood by the blood–brain barrier and the blood–nerve barrier. These barriers are composed of specialized vasculature consisting of endothelial cells with tight junctions and are impermeable to many soluble substances and restrict lymphoid and mononuclear cell migration into the nervous system. However, it is becoming increasingly evident that there are interactions between the two systems (*1–8*). For example, cells of the immune and nervous systems share common receptors (*9*), and activated T-cells can cross the blood–brain barrier (*10*). A breakdown of the barriers, allowing immune cells access, may occur with central nervous system (CNS) and peripheral nervous system (PNS) injury. Although it is known that lead affects both the immune and nervous systems, little attention has focused on how lead may affect interactions between these systems.

Preliminary work in our laboratory shows that lead exposure induces autoantibody titers to nervous system proteins, including myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) (*11,12*). The production of antibodies usually depends on both B-cells and T-cells recognizing the antigen (*13*). Immunization with protein antigens leads to the activation of different types of T-helper (Th) cells: Th1 and Th2 (*14*). These subsets differ with respect to cytokines secreted and cells activated. Some antigens can also activate B-cells independent of T-cells (*14*). There is evidence that lead can enhance immune processes (*15*) which lead to the production of autoantibodies. For example, there is evidence that lead directly activates B-cells (*16*) and increases B-cell/Th2 cell interactions which, in turn, may lead to disregulated B cell responses and autoimmune reactions (*17*).

We hypothesize that lead enhances the immunogenicity of neural proteins and thereby induces an autoimmune response to proteins of nervous system origin. To test this hypothesis, we immunized mice with native or lead-altered MBP or GFAP and measured the levels of antibodies produced in the sera of these mice using ELISA. Supernatants from lectin-stimulated splenocytes were also examined for antibody titers against MBP and GFAP and for IL-2 and IL-6 levels. IL-2 is secreted by Th1 cells and performs a number of functions including promoting the secretion of antibodies by activated B-cells (*14*). IL-6 is secreted by a number of immune-responsive cells and is believed to play a role in the differentiation of B-cells to antibody-producing plasma cells (*14*).

## Methods

Female CBA/J mice (Jackson Laboratories, Bar Harbor, Maine), 10–12 weeks old, were quarantined for 2 weeks and acclimated to a 12-hr light/dark cycle. The mice were housed in groups of three in polycarbonate boxes containing wood-chip bedding with stainless-steel lids. Animals were randomly distributed in three groups of six mice per group. Food (Purina Rodent Chow, Ralston Purina, St. Louis, Missouri) and distilled water were provided *ad libitum*.

We immunized mice with protein or lead-altered protein in 0.9% saline. The proteins used were MBP (Sigma, St. Louis, Missouri) and GFAP (IBL Research, Cambridge, Massachusetts). Lead-altered proteins were produced by incubating, with gentle agitation, a 1:100 molar ratio of native protein with lead acetate (PbOAc) in saline at 2–8°C for 24 hr. Untreated native proteins were handled in an identical manner (i.e., they were incubated in saline with gentle agitation for 24 hr at 2–8°C). For MBP, protein solutions were passed through a Sephadex column (Pharmacia, Piscataway, New Jersey) to remove any unbound lead, and these solutions were used for immunizations. GFAP solutions were not passed through a Sephadex column because preliminary studies demonstrated a significant reduction in protein recovery. Instead, a fourth

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group of mice was added to the protocol and treated with the same amount of PbOAc used to alter the GFAP. Mice received intraperitoneal inoculations on days 0, 14, and 28 with either saline, PbOAc (GFAP protocol only), 25 µg native protein, or 25 µg lead-altered protein. Ten days after the last injection, animals were anesthetized and blood was removed via cardiac puncture. Serum was obtained from the blood and stored at -70°C until use in ELISAs. Spleens were also removed and used to make single-cell suspensions for lymphoproliferative assays and for the production of lectin-stimulated supernatants.

Spleens were disrupted by forcing them through stainless-steel sieves into medium 199 (M199; BioWhittaker, Walkersville, Maryland). Clumps were allowed to settle, and the cells were washed once. We counted the cells and resuspended them in M199 supplemented with 5% fetal bovine serum, 1% 2-mercaptoethanol solution, 1% nonessential amino acids, 1% sodium pyruvate, 0.2% gentamycin, and 0.1% L-glutamine. Cells were cultured at $2 \times 10^6$ cells/ml in flat-bottomed 96-well microtiter plates (Falcon Plastics, Becton Dickson, Lincoln Park, New Jersey) for the lymphoproliferative assays and in flat-bottomed 24-well microtiter plates (Flow Laboratories, McLean, Virginia) for the production of cell-free supernatants.

Proliferative responses to the B-cell mitogen lipopolysaccharide (LPS; Sigma) and T-cell mitogen concanavalin A (Con-A, Sigma) were determined using MTT-tetrazolium (Sigma). Splenocytes were incubated in the presence of LPS (100 µg/ml) or Con-A (16 µg/ml) at 37°C in a fully humidified 5% $CO_2$ atmosphere. At 72 hr, we added MTT-tetrazolium to all wells and incubated the cells an additional 4.5 hr. We added 10% SDS-0.01 N HCl to each well and incubated the plates overnight at 37°C. The plates were then read in a microtiter plate reader at 600 nm.

Cell-free supernatants were obtained from lymphocytes stimulated with either Con-A or LPS. After 48 hr (Con-A) or 72 hr (LPS), cells were centrifuged at 1500 rpm for 20 min, and cell-free supernatants were collected. Supernatants were frozen at -70°C until used in ELISAs.

Antibody assays were conducted using serum samples and supernatant samples from LPS-stimulated splenocytes. IgG and IgM ELISAs for anti-MBP or anti-GFAP were performed. Additionally, antikeratin titers were measured by ELISA to ensure that the antibodies produced were target specific. Antigen (MBP, GFAP, or keratin) was used to coat Immulon 2 flat-bottomed microtiter plates (Fisher, Pittsburg, PA). We washed plates to remove free antigen and blocked antigen with 0.5% nonfat dry milk solution to prevent nonspecific binding of proteins. Plates were washed and serial dilutions of standards (monoclonal mouse anti-MBP or anti-GFAP; Boehringer-Mannheim, Indianapolis, Indiana) or samples were incubated in antigen-coated wells. After the incubation period, we washed unbound proteins away and added affinity-purified alkaline phosphatase conjugated anti-mouse IgG (H+L) or IgM (µ) (Jackson Immunoresearch, West Grove, Pennsylvania) to the plates. After incubation and subsequent washing, *p*-nitrophenylphosphate in diethanolamine buffer (Bio-Rad, Hercules, California) was added. We stopped the color reaction with 0.4 N NaOH solution and read the plates in a microtiter plate reader at 405 nm. For each assay, standard curves were generated using commercially available, pure monoclonal antibodies against MBP and GFAP. The ranges of the 8-point standard curves were 0–200 ng/ml. Linear regressions were fit to the curves by least-squares analysis. Since only IgG standards were available, the IgM concentrations are reported as IgG equivalents. Antibody concentrations were determined by subtracting blank optical density from a given sample optical density and inserting the resultant value into the corresponding standard equation.

Cell-free supernatants from Con A-stimulated splenocytes were analyzed for IL-2 using the Intertest 2X mouse ELISA kit (Genzyme Corporation, Cambridge, Massachusetts). IL-6 levels were quantified in cell-free supernatants from LPS-stimulated splenocytes. High binding ELISA plates (Fisher) were coated with purified murine anti-IL-6 (Pharmingen, San Diego, California) and incubated overnight at 0–10°C. After washing and blocking, standards/samples were added and incubated overnight at 0–10°C. After washing, plates were incubated with biotinylated murine anti-IL-6 (Pharmingen). Plates were washed again and incubated with avidin-peroxidase. After subsequent washing, ABTS substrate (Sigma) was added. The color reaction was stopped with sodium dodecyl sulfate in *N,N*-dimethylformamide and read in a microtiter plate reader at 405 nm.

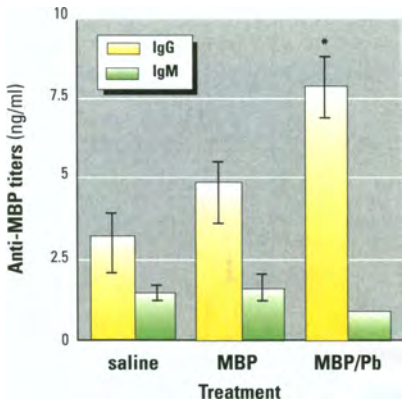


**Figure 1.** Antimyelin basic protein (MBP) titers (mean ± SE) in sera of mice ($n$ = 6/group) immunized with MBP and lead-altered MBP. Mice treated with lead-altered MBP show sera anti-MBP IgG titers that are significantly different from control (*$p$ = 0.01) and native MBP-immunized mice (*$p$ = 0.05). For this and all pertinent figures, IgM concentrations are reported as IgG equivalents.

Data were analyzed by ANOVA followed by the Dunnett's $t$-test for multiple comparisons (*18*). Statistical significance was accepted at the $p \leq 0.05$ level.

## Results

Mice were immunized with saline, MBP or lead-altered MBP (MBP/Pb) and anti-MBP antibodies were detected by ELISA in serum or cell-free supernatant of LPS-stimulated splenocytes 10 days after the last immunization. Anti-MBP titers detected in the sera of these mice are shown in Figure 1. Mice treated with lead-altered MBP had sera anti-MBP IgG titers that were significantly different from both control ($p$ = 0.01) and native MBP immunized mice ($p$ = 0.05). Mice immunized with native MBP did not show a significant increase in titers over control. For all groups, IgM titers were not different from control levels. There were background levels for both IgG and IgM titers of anti-MBP antibodies in control animals.

Anti-MBP antibodies were also determined in the LPS-stimulated splenic supernatants (Fig. 2). Mice immunized with lead-altered MBP showed supernatant anti-MBP IgG titers that were significantly different from control ($p$ = 0.05) but not native MBP immunized mice. Unlike the sera responses, anti-MBP IgM titers were higher than IgG titers in the supernatants isolated from LPS-stimulated splenocytes. However, significantly elevated IgM titers were not detected in any group.

Proliferative responses to both Con-A and LPS mitogens were determined using the MTT assay. As shown in Figure 3, mice immunized with lead-altered MBP exhibited significant increases in Con-A-induced T-cell proliferation ($p$ = 0.01) as compared to control. A significant increase was not seen in mice immunized with native MBP. There were no treatment-related increases in LPS-induced B-cell proliferation in any group.

Levels of IL-2 and IL-6 were quantified in supernatants of lectin-stimulated splenocytes. Mice immunized with lead-altered MBP exhibited increased IL-6 levels that were significantly different from control ($p$ = 0.05). However, the levels of IL-2 produced by stimulated splenocytes

from these animals were not different from control values (Fig. 4).

Immunizations were also conducted with another lead-altered neural protein, GFAP. The treatment protocol used for GFAP differed from that used for MBP in that a group of mice exposed to PbOAc alone was added. This was done because recovery of lead-altered GFAP from the Sephadex columns was poor. GFAP-immunized mice exhibited responses in the sera that were analogous to those observed in the sera of MBP-immunized mice. Mice immunized with lead-altered GFAP showed sera anti-GFAP IgG titers that were statistically different from control ($p$ = 0.01), lead alone ($p$ = 0.01) and native GFAP-immunized mice ($p$ = 0.05). There were no elevated titers of anti-GFAP IgM in the sera (Fig. 5).

Lectin-stimulated supernatants from GFAP-immunized mice were also analyzed for the production of anti-GFAP antibodies and for IL-2 and IL-6 levels. The only observed changes occurred in mice treated with native GFAP. These animals exhibited supernatant anti-GFAP IgG titers that were significantly different from controls ($p$ = 0.01). Mice immunized with lead-altered GFAP did not show an increase in anti-GFAP IgG titers. In addition, there were no increases in anti-GFAP IgM titers in any group (Fig. 6). Stimulated splenocytes taken from mice treated with lead-altered GFAP showed no increases in either IL-2 or IL-6 production (Fig. 4). The lymphoproliferation assays showed no increases in the proliferation of splenocytes stimulated with either Con-A or LPS (data not shown).

Serum antibodies did not react with keratin for any of the animals in any group, confirming that the ELISA reactions were target specific (data not shown).

## Discussion

The present study shows that lead enhances the immunogenicity of two nervous system proteins, MBP and GFAP. These results support our hypothesis that the progression of lead-induced neurotoxicity may be due to the production of autoantibodies against neural proteins provoked by a lead-induced enhancement of the immunogenicity of those proteins. These results also complement our earlier work showing that lead exposure induces autoantibody titers against nervous system proteins, including MBP and GFAP (*11,12*).

Usually, the first immmunoglobulin to occur after initial antigen contact is IgM (*13*). In many T-cell independent responses, IgM remains the primary immunoglobulin. However, isotype switching to IgG occurs during T-dependent immune responses (*13*). In this study, enhanced IgG antibody titers were seen in the sera, whereas IgM titers in the sera were unchanged from control values. This indirect evidence suggests a T-cell dependent response for the generation of antibodies against both lead-altered MBP and GFAP.

MBP is a negatively charged 18 kDa protein of approximately 170 amino acid residues (*19*). It is distinctive for its lack of sulfhydryl groups (*19*). In contrast, GFAP is a positively charged 52 kDa protein which contains methionine and cysteine groups (*20*). These differences between the proteins suggest that lead may be binding and altering the immunogenicity of each in a different manner. The lead ion carries a double positive charge and may bind to MBP in a charge neutralization process. Because of the double positive charge, it is also possible for lead to cross-link between negative charges on two different MBP molecules making the lead-altered form more immunogenic in this manner. In contrast, because GFAP is positively charged but contains sulfhydryl groups, it is possible that lead is binding to a methionine or cysteine residue and perhaps unmasking an epitope, making the lead-altered form of GFAP more immunogenic. These potential differences in the way lead binds to MBP and GFAP may produce different types of antigenic determinants on the two lead-altered proteins causing antibodies to be generated against the two lead-altered proteins through different mechanisms. The observed differences between the responses generated by the lead-altered forms of MBP and GFAP in the supernatants of lectin-stimulated splenocytes provide evidence for this proposition.

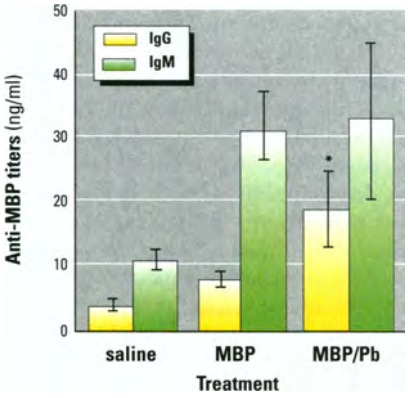


**Figure 2.** Antimyelin basic protein (MBP) titers (mean ± SE) ($n$ = 6/group) in lipolysaccharide (LPS)-stimulated splenic supernatants of mice immunized with MBP and lead-altered MBP. Mice treated with lead-altered MBP show supernatant anti-MBP IgG titers that are significantly different from control mice (*$p$ = 0.05).

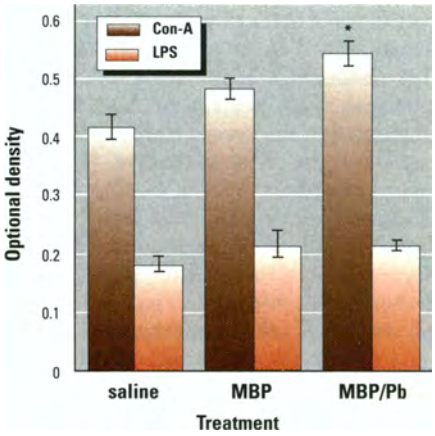


**Figure 3.** Lymphoproliferation in mice (mean ± SE) ($n$ = 6/group) immunized with myelin basic protein (MBP) and lead-altered MBP. Mice treated with lead-altered MBP exhibit significant increases in Con-A induced T-cell proliferation (*$p$ = 0.01) compared to control.

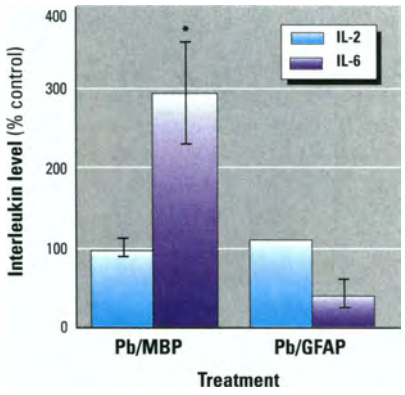


**Figure 4.** Interleukin (IL)-2 and IL-6 levels (mean ± SE) ($n$ = 6/group) in lectin-stimulated splenocyte supernatants from mice immunized with either lead-altered myelin basic protein (MBP) or lead-altered glial fibrillary acidic protein (GFAP). Mice treated with lead-altered MBP show supernatant IL-6 levels that are significantly different from control mice (*$p$ = 0.05).

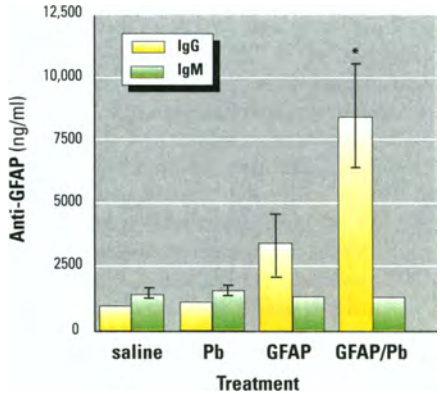


**Figure 5.** Antiglial fibrillary acidic protein (GFAP) titers (mean ± SE) in sera of mice ($n$ = 8/group) immunized with GFAP and lead-altered GFAP. Mice treated with lead-altered GFAP show sera anti-GFAP IgG titers that are significantly different from control (* $p$ = 0.01) and lead alone (*$p$ = 0.01), and native GFAP-immunized mice (*$p$ = 0.05).

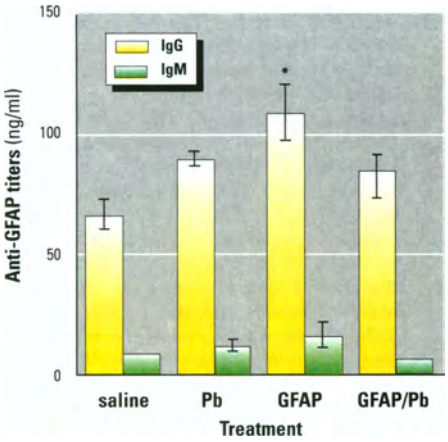


**Figure 6.** Antiglial fibrillary acidic protein (GFAP) titers (mean ± SE) ($n$ = 6/group) in lipopolysaccharide (LPS)-stimulated splenic supernatants of mice immunized with GFAP and lead-altered GFAP. Mice treated with native GFAP show supernatant anti-GFAP IgG titers that are significantly different from control mice (*$p$ = 0.01).

In the splenic supernatants, Con-A-induced proliferation was increased in mice treated with lead-altered MBP (Fig. 3) but not with lead-altered GFAP (data not shown). Further, there was a significant increase in IL-6 production by LPS-stimulated splenocytes of mice immunized with lead-altered MBP but not with lead-altered GFAP (Fig. 4). In addition, anti-MBP IgG antibody titers were elevated in the supernatants of LPS-stimulated splenocytes of mice immunized with lead-altered MBP, but an analogous response was not seen in the splenocyte supernatants of mice immunized with lead-altered GFAP (Figs. 2 and 6). Since there is indirect evidence that both lead-altered proteins induce antibody production through T-cell-dependent mechanisms, the splenic supernatant responses suggest that different subsets of T-helper cells may be involved in the generation of antibodies by the two different lead-altered proteins.

It is known that immunization with protein antigens leads to the activation of different types of helper T-cells: Th1 and Th2 (*14*). Th2 cells are considered optimal helpers for B-cell responses, while Th1 cells are not considered efficient helpers (*17*). These two subtypes differentially regulate immune responses via the cytokines they secrete. Th1 cells secrete IL-2 and inteferon-g, whereas Th2 cells secrete IL-4, -5, -6, and -10 (*21*). Of these cytokines, IL-2 and IL-6 were selected for analyses in the present studies because they stimulate antibody secretion by B-cells (*22*). Our evidence suggests that lead-altered MBP activates different cytokine-secreting cells from lead-altered GFAP, as elevated IL-6 levels were found in the splenic supernatants of lead-altered MBP-treated mice but not in the supernatants of lead-altered GFAP-treated mice.

Because the present study demonstrates that lead enhances the immunogenicity of two neural proteins, it raises the possibility that lead, and perhaps other metals, may be involved in the pathogenesis of immunologically mediated neurological diseases. Several of these diseases manifest autoantibodies to neural proteins at some stage of the disorder. For example, in both humans and experimental animals, MBP is the target antigen for immune processes leading to autoimmune encephalomyelitis (*23*). Autoantibodies against MBP as well as GFAP have been found in the cerebrospinal fluid (CSF) of multiple sclerosis patients (*24,25*). In addition, stimulated B-cells from the CSF of multiple sclerosis patients produce antibodies against MBP (*26*). Autoantibodies against MBP and GFAP are also observed in other disorders. For example, increased anti-MBP titers are seen in patients with subacute sclerosing panencephalitis and post-infectious encephalomyelitis (*27*). Patients with Lyme neuroborreliosis also exhibit elevated CSF anti-MBP titers (*28*). Autoantibodies against MBP as well as against GFAP are seen in the serum of patients with Alzheimer's disease (*29–31*). Although Alzheimer's disease primarily affects neurons, there is secondary involvement of myelin; therefore, humoral immune reactions to MBP might develop as the disease progresses (*29*). In addition, GFAP has been shown to stimulate proliferation and immunoglobulin synthesis by lymphocytes from patients with Alzheimer's disease (*4*). Serum anti-GFAP autoantibodies are seen in patients suffering from senile dementias and in healthy, aging people (*32*). Because of the presence of autoantibodies against nervous system proteins in several neurological disorders and because of the suspected involvement of environmental pollutants in the pathogenesis of neurological diseases, it is reasonable to propose a mechanism whereby metal alteration of neural proteins leads to autoantibody production against neural structures, causing progressive degeneration of the nervous system.

Finally, it should be pointed out that autoantibodies against neural proteins have the potential to serve as early biomarkers of impending neurological disease because they may appear before traditional pathological manifestations. If our hypothesis is correct and metals induce or intensify immune responses against neural structures, neurotypic autoantibodies have the potential to act as early and sensitive biomarkers for the effects of neurotoxic metals. Studies concerned with the timing of metal-induced neurotoxicity versus the timing of the appearance of neurotypic antibodies would address this question and will be the subject of a future report.

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