

Assessment of metallothionein and antibodies to metallothionein in normal and autistic children having exposure to vaccine-derived thimerosal

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Allergic autoimmune reaction after exposure to heavy metals such as mercury may play a causal role in autism, a developmental disorder of the central nervous system. As metallothionein (MT) is the primary metal-detoxifying protein in the body, we conducted a study of the MT protein and antibodies to metallothionein (anti-MT) in normal and autistic children whose exposure to mercury was only from thimerosal-containing vaccines. Laboratory analysis by immunoassays revealed that the serum level of MT did not significantly differ between normal and autistic children. Furthermore, autistic children harboured normal levels of anti-MT, including antibodies to isoform MT-I (anti-MT-I) and MT-II (anti-MT-II), without any significant difference between normal and autistic children. Our findings indicate that because autistic children have a normal profile of MT and anti-MT, the mercury-induced autoimmunity to MT may not be implicated in the pathogenesis of autism.

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Key words: autism; metallothionein; autoimmunity; mercury; thimerosal; immunotoxicity

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Accepted 4 October 2005

Autism is a biological disorder that impairs the functioning of the brain in children, affecting their ability for social interaction, language, imagination, communication and cognition. The aetiology and pathogenesis of the disorder are not well known but the immune factors such as autoimmunity to brain might play a pathogenetic role in autism (1–4). Although the trigger agent for autoimmunity is not well known, there is serological evidence for a virus infection (5–7). Heavy metals like mercury are also capable of eliciting autoimmune reaction but a recent study of autoimmune markers in autism did not support this possibility (8). In the body, the biological response to heavy metals is exerted by metallothionein (MT), which is a cysteine (Cys)-rich protein that chiefly transports heavy metals for detoxification purposes. The MTs are a family of proteins that are inducible by a variety of environmental factors and stress conditions

(9–13). The mammalian MT is characterized by a molecular weight of 6–7 kD, containing 60–68 amino acid residues, among them 20 Cys, and binding a total of seven equivalents of bivalent metal ions. Because of diverse biochemical properties of MT proteins, we hypothesized that if the children with autism have heavy metal or mercury toxicity then they might also have up-regulation of MT and anti-MT as a sign of metal-induced autoimmune process. Therefore, we conducted present study to assess the status of MT and anti-MT as biomarkers of detoxification response to vaccine-derived mercury in children with autism. MT is known to exist in four isoforms, the ubiquitous MT-I and MT-II, the brain specific MT-III, and the squamous epithelium specific MT-IV (9, 14, 15), but in the present study we screened antibodies against MT, MT-I and MT-II proteins only because of their availability from commercial sources.

Materials and methods

Subjects in the study

The study included 62 autistic children (aged 6.7 ± 3.8 yr) and 60 normal children (aged 7.4 ± 3.8 yr). As described previously (1–4), the clinical diagnosis of autism was essentially according to the standard DSM-IV criteria of the American Association of Psychiatrists, Washington, DC. The Institutional Review Board (IRB) of Utah State University reviewed and approved our research protocol that involved the use of human blood specimens. At the time of blood draw, all children were at the baseline without any prescription medications or biomedical interventions. All autistic and normal children had received their full immunization schedule with multiple vaccines, including cellular pertussis vaccine containing diphtheria–tetanus–pertussis (DTP), hepatitis B (Hep B) vaccine, inactivated polio vaccine (IPV), haemophilus influenzae b (Hib) vaccine, and measles–mumps–rubella (MMR) vaccine. Therefore, as far as we know, the children in the study were exposed to mercury from thimerosal-containing vaccines only but not to any other source of the mercury. We used previously collected serum specimens (1–4) that were stored frozen at -20°C .

Quantification of metallothionein (MT)

This was performed by an enzyme-linked immunosorbent assay (ELISA) method according to our published reports (6, 8). The method involved coating of the microplate with rabbit polyclonal antibodies to MT ($50\ \mu\text{l}/\text{well}$ of pre-diluted commercial stock from Biosense Laboratories, Bergen, Norway). The blocking was carried out with 1% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS). After five washings with PBS-Tween-20 (0.5%), the reference MT or serum samples ($50\ \mu\text{l}/\text{well}$) were incubated overnight in the fridge. The plates were washed in the same fashion, followed by the addition of the mouse monoclonal antibody to MT ($50\ \mu\text{l}/\text{well}$ of pre-diluted commercial stock from MP Biomedicals, Aurora, OH, USA). After 90 min incubation at room temperature, the plates were washed as above. Then the plates were incubated for 90 min at room temperature with the goat antimouse-IgG-alkaline phosphatase ($50\ \mu\text{l}/\text{well}$ of pre-diluted commercial stock from Sigma-Aldrich, St Louis, MO, USA). The plates were washed again as above, followed by the addition of $50\ \mu\text{l}/\text{well}$ of the substrate solution that was a 1 mg/ml solution of *p*-nitrophenylphosphate prepared in the same fashion as before

(6, 8). After 110 min of colour reaction at room temperature, the absorbance readings were taken at 405 nm using the Model EL311 Bio-Tek Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). After the subtraction of blank absorbance reading, the amount of MT protein was calculated in reference to an MT standard and expressed as the amount (μg) per millilitre of the serum.

Assay for antibodies to metallothionein (anti-MT)

The detection of anti-MT was performed according to our published ELISA method (6, 8) except that the screening antigen was MT, MT-I or MT-II protein (Sigma-Aldrich). Briefly, the microplates were coated with $4\ \mu\text{g}/\text{well}$ of MT, MT-I or MT-II, followed by blocking with BSA and four washings with PBS-Tween (0.5%) buffer. Pre-diluted human sera ($100\ \mu\text{l}/\text{well}$) were incubated for 1 h at room temperature, followed by four washings with PBS-Tween buffer. Then $100\ \mu\text{l}/\text{well}$ of goat antihuman-IgG-alkaline phosphatase (1:1,000 diluted) was incubated at room temperature for 1 h. The plates were washed again and $100\ \mu\text{l}/\text{well}$ of the substrate solution (1 mg/ml solution of *p*-nitrophenylphosphate) was added. After 30 min at room temperature, the absorbance readings were taken at 405 nm using the Model EL311 Bio-Tek Microplate Reader. After the subtraction of blank absorbance reading, the level of anti-MT was calculated in terms of EIA Units arbitrarily defined as one optical density unit is equal to 100 EIA Units.

Data analysis

The data are presented as the average of the group $\pm$ standard error of the arithmetic mean (s.e.m.). The statistical significance ($p \leq 0.05$) was evaluated by the Student *t*-test using the STAT VIEW software for Macintosh.

Results

The serum profiles of MT protein and anti-MT are shown in Figs. 1–3. Firstly, the experimental data for MT in sera of normal and autistic children are depicted in Fig. 1. Although the MT protein was found in both populations of children, its concentration in the serum did not differ significantly ($p = 0.792$) between the normal and autistic children. Secondly, the determination of antibodies to MT was carried out first to study the effect of serum dilution (Fig. 2). Using MT as the screening antigen, we found the

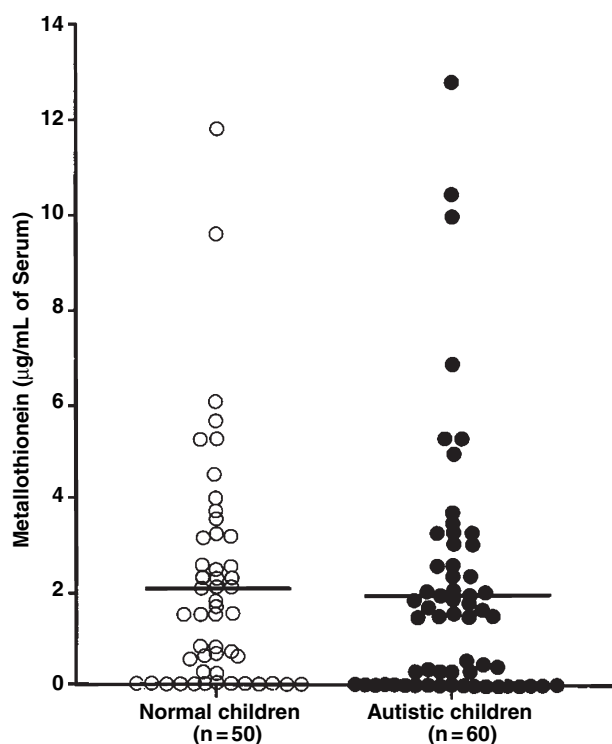


Fig. 1. Serum level of metallothionein (MT) in normal and autistic children. MT protein was quantified in sera of normal children (open circles, $n = 50$) and autistic children (solid circles, $n = 60$) by ELISA method. As depicted here, there was no significant difference in the serum level of this protein in two subject populations ($p = 0.792$).

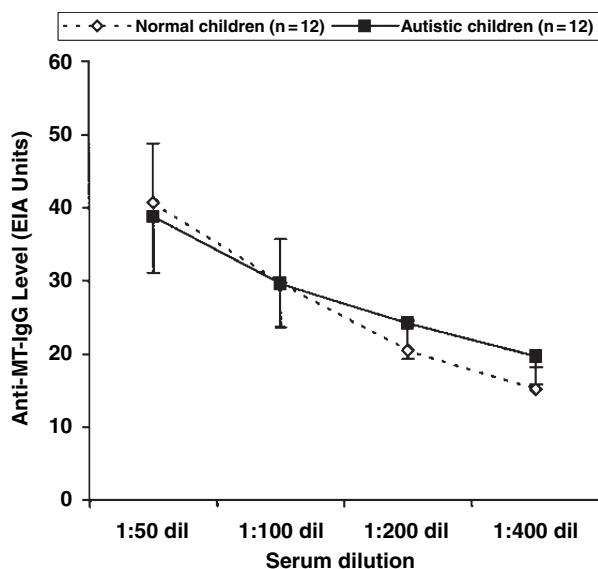


Fig. 2. Influence of serum dilution on antibodies to metallothionein (MT) in normal and autistic children. Antibodies to MT were detected in sera of normal children (dotted line, $n = 12$) and autistic children (solid line, $n = 12$) at four dilutions, namely 1:50, 1:100, 1:200 and 1:400. The data are given as the arithmetic mean $\pm$ standard error (s.e.m.). Note that there were no significant differences between the normal and patient groups (p -values were ≥ 0.385 at all four dilutions).

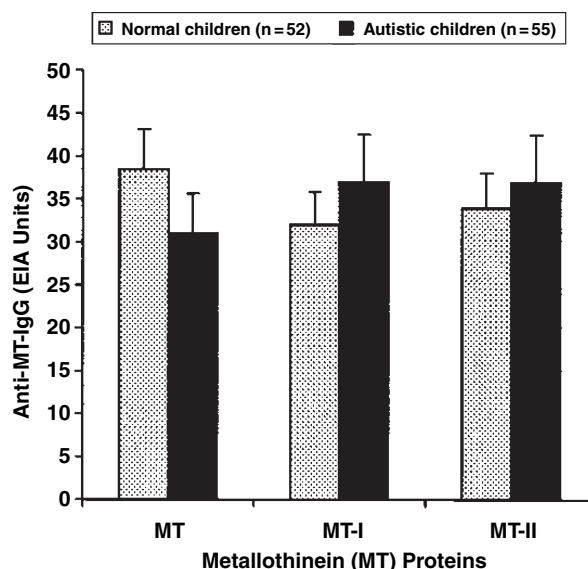


Fig. 3. Distribution of antibodies to metallothionein (MT) proteins in normal and autistic children. Antibodies to MT proteins were screened in sera of normal children (dotted bars, $n = 52$) and autistic children (solid bars, $n = 55$). Serum samples were pre-diluted to a 1:50 dilution before the immunoassay. Note that there were no significant differences between the normal and patient groups (p -values were ≥ 0.1 for anti-MT, -MT-I and -MT-II).

presence of anti-MT in both normal and autistic children. At four serum dilutions (1:50, 1:100, 1:200 and 1:400 dilutions), autistic children harboured about the same level of anti-MT as the normal children did (Fig. 2) and the difference between these two groups did not attain statistical significance (p -values being ≥ 0.385 at all four dilutions). Not included here but the effect of serum dilution was about the same when MT-I and MT-II were used as the screening antigens. Because a serum dilution of 1:50 showed the highest level of antibody, it was subsequently used to screen all other sera of subjects in the study. The outcome of this screen is shown in Fig. 3, which contains quantitative levels of anti-MT, -MT-I and -MT-II. The serum level of anti-MT proteins in autistic children did not change significantly (p -values being ≥ 0.1) when compared with their levels in normal children (Fig. 3). Thus, there was no significant difference in the distribution of antibodies to MT proteins in normal and autistic children.

Discussion

Metallothioneins are ubiquitous low molecular weight proteins. Owing to their extremely high metal and sulphur content, they are thought to play a multitude of biological roles, including the neutralization of heavy metal toxicity and

protection from stress conditions (9–15). Although the mercury is widespread in the environment, humans are exposed to mercury mainly through the consumption of contaminated fish and medicinal products such as vaccines, dental amalgams and skin ointments. As the childhood vaccines contain mercury derivative thimerosal as a preservative, they represent the primary source of mercury exposure to children. Mercury is a well-known environmental agent with toxic effects on just about all biosystems, including the nervous system and the immune system (16–21).

Mercury from thimerosal-containing vaccines has recently been suspected as a risk factor for neurodevelopmental disorders, including autism. Anecdotal, the mercury poisoning was speculated to resemble autistic disorder, implying that the neurological manifestations of autism originate from the exposure to thimerosal-derived mercury (22). However, a careful literature review by neurologists showed that the neurological manifestations of autism did not resemble the clinical signs of mercury poisoning, thus precluding an aetiological link between thimerosal and autism (23). Moreover, there is very little, if any, laboratory-based experimental research on this topic. Because mercury is well known to influence immune system, people generally tend to think that mercury is always immunotoxic. This could be misleading because mercury also exerts positive effects such as lymphoproliferation (24). Fundamentally, there is very little research on mercury-induced immune regulation and autoimmunity but this topic needs to be investigated with scientific rigour. As mercury can induce autoimmunity (19–21), we explored the possibility that mercury could trigger autoimmunity in autism also. However, this may not be the case because of the novel findings as discussed below.

Investigations of faulty immune regulation in autism and mercury-induced immune abnormalities have revealed some important differences between these two conditions. The mercury-induced autoimmunity is generally typified by the presence of circulating autoantibodies, in particular the anti-nuclear (anti-nucleolar) antibodies and anti-laminin antibodies (20, 21). This autoimmune reaction is rare in man but when it occurs it causes nephrotic syndrome (21). As far as we know, children with autism do not have nephrotic syndrome and they do not harbour abnormal titres of anti-nuclear (anti-nucleolar) antibodies and/or anti-laminin antibodies (8). Moreover, the people with mercury-induced autoimmunity have auto-antibodies to the

low-molecular weight (68 kD) neurofilament protein (19) whereas the antibodies to this antigen are virtually absent in autistic children (2). In contrast, however, the children with autism harbour autoantibodies predominantly to brain myelin basic protein (MBP) (1, 2), which are rarely detected in individuals exposed to mercury (19). Furthermore, autism involves a Th₁ immune response (3) whereas the mercury exposure induces a Th₂ response (25). Immunologically, therefore, the two conditions demonstrate some important differences, which could imply that mercury is not likely the environmental agent for setting up the autoimmune reaction in autism.

Until recently, antibodies to MT were not known to exist in man. Jin et al. (26) recently showed the occurrence of these antibodies in the human population. They studied anti-MT in sera of 39-year-old Japanese population comprised of about twice the number of females than males. They found the presence of anti-MT in approximately 4% of healthy adults, 6% of adult patients with atopic dermatitis, and 51% of adult patients with atopic dermatitis after metal allergy that was experimentally induced against predominantly nickel and to a much lesser degree mercury also. These researchers concluded that the exposure to metals induces the synthesis of antibodies to MT in adult patients suffering from atopic dermatitis. However, the pathological significance of these antibodies remains unclear at the present time.

As described here, we conducted the first ever study of serum profiles of MT protein and anti-MT in autistic children who might show up-regulation of these biomarkers if the exposure to mercury was related to autoimmunity in autism. The outcome of this study clearly showed that there was no significant difference in autistic and normal children as far as the MT and anti-MT serum profiles are concerned. Both populations had almost the same profile for these two biomarkers, a result that might be expected because both normal and autistic children have had their immunizations in compliance with the US government policy. Now, there are at least two different lines of studies (present study and Ref. 8) that did not render support for a mercury and autoimmunity connection in autism. But, these negative findings alone are probably not sufficient to rule out such a relationship completely. While more experimental research is necessary on this topic, we conclude that the chance of setting up an autoimmune response from exposure to small amounts of vaccine-derived mercury is rather rare, unless of course

some other factors concurrently contribute and increase the susceptibility to mercury-induced autoimmunity. The other factors might include bacterial toxins, toxic chemicals or dietary peptides, which by binding to lymphocyte receptors and tissue enzymes could potentially be relevant to autism (27). But because of the lack of disease-specific correlations, the significance of these factors remains to be established.

Conceptually, autism is generally considered to be a multi-factorial disorder. To this end, it is instructive to explore and pay attention to other causative mechanisms for autism. One such mechanism could be the virus-induced autoimmune pathogenesis (1–4) that was demonstrated by a specific serological association between brain MBP and measles virus but not between other brain proteins and other viruses in autism (2, 4–7). The MBP antibody and the measles virus antibody were the only two parameters that showed a positive correlation in >90% of the autistic children (5, 6), but none of the other autoimmune parameters investigated so far has shown any correlation that might be of pathological relevance to autism. This observation might be an important clue to identifying viral aetiology of this brain disorder. Accordingly, a novel protocol has recently been developed for testing virus serology and brain-specific autoimmunity in autistic patients, who might also draw benefits of the immune modulation therapy (4). Based on the negative findings of the present study and that of our previous study (8), we conclude that the vaccine-derived thimerosal is probably not a contributing factor for autoimmunity in autism. While one such factor might be a virus (4–6), we think that more laboratory-based research is needed to identify the agent for setting off the autoimmune process in autism.

Acknowledgments

This work was supported by charitable contributions from the Autism Research Institute, BHARE Foundation and Dudley T. Dougherty Foundation. The authors did not have any conflict of interest in this regard. Jeff Hanson was a pre-med student.

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