Peer reviewed article

Anti-Metallothionein IgG and levels of metallothionein in autistic families

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Summary

Metallothioneins (MTs) are a family of small proteins containing 61–68 amino acids with an unusually high concentration of cysteine. MT-1, the most functional and active MT in humans, has the ability to react with and enhance the detoxification of a number of metals including zinc, mercury, copper and cadmium. MT dysfunction may result, then, in many of the aetiological syndromes observed in autistic children, such as the leaky gut. It has been proposed that allergic autoimmune reactions occurring after exposure to heavy metals, may contribute to some symptoms associated with autism. Therefore abnormalities in MT concentration and/or structure, as well as the presence of anti-MT antibodies, may be associated with autism.

We used direct ELISAs to quantitate the concentration of serum anti-metallothionein IgG in 66 individuals (parents and children) from 14 families with autistic children, as well as 11 controls from families with no history of autism. We measured the concentration of serum metallothionein in 39 of the above family members from 8 families. Our results indicate that a significantly high number (23 of 66) of autistic family members had high levels of anti-metallothionein IgG, when compared to controls (10) and the production of these antibodies correlated with levels of metallothionein, suggesting that the production of these antibodies is inherited. However, the presence of these antibodies does not correlate with autism, types of autism, including regression, or demographics such as allergies, respiratory problems or GI disease. This suggests that the presence of anti-metallothioein antibodies is not causative to autism and may be the result of other immunological pathology seen in many autistics.

Key words: autism; metallothionein; anti-metallothionein antibodies

Introduction

Autistic Spectrum Disorder (ASD) is a neurodevelopmental syndrome with onset prior to 36 months of age. Diagnostic criteria consist of impairments in sociality and communication plus repetitive and stereotypic behaviours [1]. Traits strongly associated with autism include movement disorders and sensory dysfunctions [2]. Although autism may be apparent soon after birth, many autistic children experience at least several months, up to a year or more in some cases, 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 recognised [5]. Primary data come from victims of contaminated fish or grain, from acrodynia induced by Hg in teething powders, and from individual instances of mercury poisoning (HgP), many occurring in occupational settings. 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 immunisations 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]. There may be an association between mercury toxicity and the onset of autism [4, 8, 9, 10].

The Metallothioneins (MT) are a family of small proteins containing 61–68 amino acids with an unusually high concentration of cysteine (30%). MT-1, the most functional and active MT in humans, has 21 cysteines. Cysteine contains a sulfhydryl group (SH) that has the ability to react with a number of metals including zinc, mercury, copper and cadmium.

Divalent metals such as copper, zinc, and manganese are toxic to cells in elemental or ionic form. These metals are "enveloped" or "bound" to the small linear MT, which supervises and regulates metal levels in blood, brain and the periphery and therefore plays a major role in heavy metal detoxification of these metals [11–14].

Control tissues provided by the National Disease Research Interchange, Philadelphia, PA.

Besides detoxification of heavy metals, functions of MT in the body include development of brain neurons, maturation of the GI tract, antioxidation, boosting immune function and delivery of zinc to cells [11, 15–17]. Evidence also suggests that Autistic individuals are prone to developing autoimmune disorders and Autism appears to be more common in families with a history of autoimmune disorders [18, 19].

MT dysfunction may result, then, in many of the issues seen with autistic children, such as the leaky gut syndrome, incomplete breakdown of casein/gluten protein by zinc dependent enzymes, disrupted ability to combat yeast, reduced production of stomach acid and impaired stimulation of the pancreas by secretin. It may also lead to inability to clear the body of heavy metals, a dysfunctional immune system, and ultimately to the neurological changes seen in ASD. It would also explain the male sex predominance (4:1) seen in autism, because MT synthesis is enhanced by oestrogen and progesterone. In a study of 503 autism-spectrum patients at The Pfeiffer Treatment Centre, scientists found abnormal levels of copper and zinc in blood ($p < 0.0001$) indicating defective functioning of metallothionein (MT) proteins [20].

Metallothionein levels fluctuate in direct response to heavy metal levels such as zinc [21]. The fluctuation of metallothionein levels and/or the production of abnormally structured metallothionein in autistic individuals may stimulate an auto-immune response and therefore, may be associated with anti-MT antibody production and the etiology of autism.

In this preliminary study we used ELISAs and Western Blotting to detect and measure serum antibodies to metallothionein in 33 autistic and 33 non-autistic individuals (parents and non-autistic siblings) from 14 families, as well as 11 age-matched control individuals with no family history of autism.

We also used direct ELISAs to quantitate the concentration of serum metallothionein in 21 autistic and 18 non-autistic individuals from 8 of the above autistic families.

Our results indicate that a significantly high number (23 of 66) of family members from families with autistic children have high levels of anti-metallothionein IgG, when compared to controls (0/11 age matched individuals from families with no family history of autism) and the production of these antibodies is likely inherited. However, the presence of these antibodies does not correlate with autism (parents and non-autistic siblings were just a likely to have anti-MT IgG), type of autism such as regression, or demographics such as allergies, respiratory problems or GI disease.

Materials and methods

ELISA (Enzyme Linked Immunosorbent Assay) to measure auto-antibodies to metallothionein

Purified metallothionein-1 (Sigma), at a concentration of 500 ng/µl of bicarbonate buffer (pH 9.6), was fixed to wells of a 96 well polystyrene microculture plate (Corning) by incubation overnight at 4 degrees C. Excess metallothionein was dumped from wells and all wells were blocked by washing 3× with 300 microliters of blocking solution (Superblock, Pierce). One hundred microliters of primary antibody (Positive control – mouse Mab to metallothionein-1 diluted 1:500 with PBS; Negative control – PBS; Experimental serum from autistic and non-autistic individuals diluted 1:500 with PBS) was added to appropriate wells and plate was incubated for 2 hours at 37 degrees C. All wells were washed 3 times using PBS/tween. One hundred microliters of goat anti-mouse secondary antibody, conjugated with alkaline phosphatase (Biorad), diluted 1:5000 with PBS to positive control; Goat anti-human (Biorad) diluted 1:5000 with PBS to all other wells, added to appropriate wells and incubated for 45 minutes at 37 degrees C. All wells washed 5 times with PBS/tween. One hundred microliters of substrate for alkaline phosphatase added to all wells and plate was incubated at room temperature until significant colour change in positive control. Colour change measured using ELISA Reader (Biorad).

Direct ELISA to quantitate the concentration of serum metallothionein

Purified metallothionein-1 (Sigma) at concentrations of 1 µg, 500 ng, 50 ng and 5 ng per 100 µl of bicarbonate buffer (pH 9.6), and serum at a dilution of 1:500, were fixed to wells of 96 well polystyrene microculture plate (Corning) by incubation overnight at 4 degrees C. Excess metallothionein/serum was dumped from wells, and all wells were blocked by washing 3× with 300 microliters of blocking solution (Superblock, Pierce). One hundred microliters of primary antibody (mouse Mab to metallothionein-1, diluted 1:500 with PBS) was added to all wells and plate was incubated for 2 hours at 37 degrees C. All wells were washed 3 times using PBS/tween. One hundred microliters of secondary antibody, conjugated with alkaline phosphatase (goat anti mouse, Biorad, diluted 1:5000 with PBS) added to all wells and incubated for 45 minutes at 37 degrees C. All wells washed 5 times with PBS/tween. One hundred microliters of substrate for alkaline phosphatase added to all wells and plate was incubated at room temperature until significant colour change in positive control. Colour change measured using ELISA Reader (Biorad).

Western blotting to detect auto-antibodies to metallothionein Protein preparation

Purified metallothionein-1 (Sigma), and control purified Myosin and Actin (BioRad) incubated 1:1 with loading dye at 95C for 5 minutes. Protein run on SDS-Page (18%) (BioRad), at 100 volts, 1 microgram/lane.

Blot

Proteins were transferred to nitrocellulose at 30 volts, overnight at 4C. Nitrocellulose flooded with blocking solution (1% casein/PBS) and rocked gently overnight.

Immunoblot

Ten ml of primary antibody (Positive control – mouse Mab to metallothionein-1 diluted 1:500 with PBS; Negative control – PBS; Experimental serum from autistic and non-autistic individuals diluted 1:500 with PBS) was flooded over appropriate nitrocellulose sheets and incubated for 2 hours at room temperature. All sheets were washed 3 times using PBS/tween, with five minutes for each wash, rocking gently at room temperature. Ten ml of secondary antibody conjugated with Horse Radish Peroxidase (HrP) (goat anti-mouse [Biorad] diluted 1:5000 with PBS to positive control; Goat anti-human [Biorad] diluted 1:5000 with PBS to all other wells) added to appropriate sheets and incubated for 45 minutes at room temp. All sheets were washed 5 times with PBS/tween, with five minutes for each wash, rocking gently at room temperature. Ten ml of substrate for HrP added to all sheets at room temperature until significant colour change in positive control.

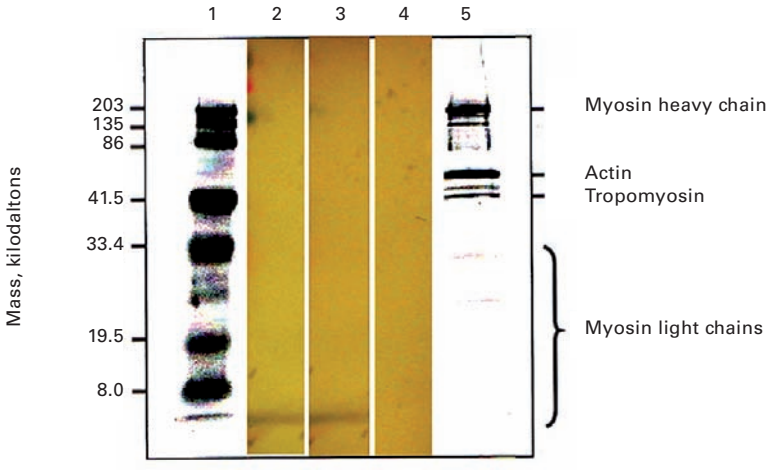
Results

The presence of anti-metallothionein IgG was confirmed using western blotting. In figure 1, antibodies from an autistic family member (973) are shown directed at metallothionein, compared to the same reactivity of monoclonal antibody to metallothionein (positive control).

We used direct ELISAs to quantitate the concentration of serum anti-metallothionein IgG in 66 individuals (parents and children) from 14 families (from Autism Genetic Resource Exchange) each family having at least one autistic child, as well as 11 age-matched individuals from families

Figure 1

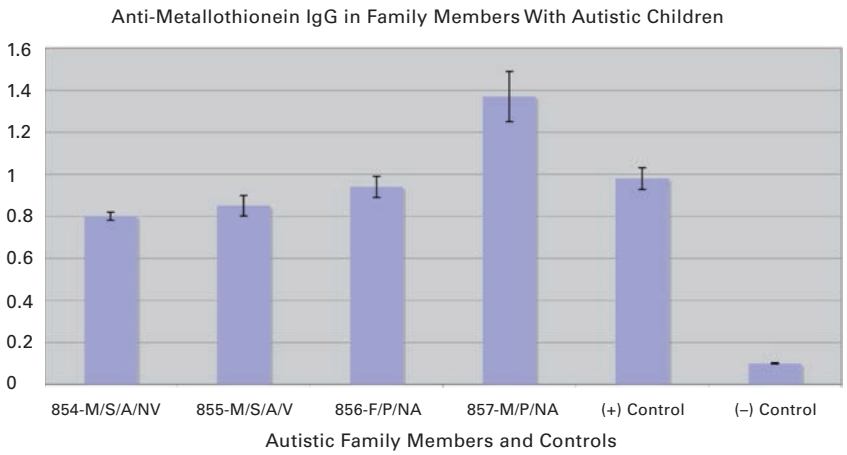
Western Blot showing anti-MT antibodies of autistic family member 937 specifically reacts with MT (approximately 7k Daltons).



Lane 1: Kaleidoscope standards
2. Positive Control (MT)
3. 973
4. Negative Control (Myosin/Actin)
5. Actin/Myosin

Figure 2

Typical ELISA demonstrating presence of anti-metallothionein IgG in family members compared to + control (monoclonal IgG to MT) and – controls (PBS).



P – Parent
S – Sibling
854 – patient ID
A – Autistic
NA – Non-autistic
V – Verbal
NV – Non-verbal
® – Regression
PDD – Prevasive Developmental Disorder
FT – Fraternal Twin
IT – Identical Twin

* 4 samples in each group/assay
** each family was tested at least twice with similar findings

Table 1

Family ID	ID #	Diagnosis	Clinical	Sex	Twins	Relationship	MT ng/ml	Anti-MT	High Ab	Resp	Resp T	Aller	Aller_T	HB	GI	GI T
AU0030	202-1	non-autistic		F		Parent	14	0.6**		0		0			0	
AU0030	203-2	non-autistic		M		Parent	14	0.45		0		0			0	
AU0030	204-3	autistic	V-Asp	M		Sibling	13	0.44								
AU0030	205-4	autistic	NV-R	M	IT	Sibling	15	0.49		0		0		0	0	
AU0030	206-5	autistic	NV	M	IT	Sibling	12	0.4		0		0		1	0	
AU0149	561-1	non-autistic		F		Parent	**51**	1.06**								
AU0149	562-2	non-autistic		M		Parent	**39**	0.6 **								
AU0149	563-5	autistic	NV-R	M		Sibling	**42**	0.6 **								
AU0149	564-4	autistic	NV-R	M		Sibling	**41**	0.48								
AU0149	565-3	non-autistic		F		Sibling	**40**	0.58								
AU0162	81-1	non-autistic		F		Parent	10	0.95 **		0		1	1		0	
AU0162	82-2	non-autistic		M		Parent	13	1.28 **		1	1	1	2		0	
AU0162	83-3	non-autistic		M		Sibling	10	1.12 **		-1		1	3	1	1	1
AU0162	84-5	autistic	NV	F		Sibling	10	0.74 **		-1		-1		-1		
AU0162	85-4	autistic	V	F		Sibling	11	0.51		-1		-1		-1		
AU0561	971-4	PDD	V	M		Sibling	**52**	0.86 **		0		0		0	0	
AU0561	972-3	autistic	NV-R	M		Sibling	**54**	0.77 **		0		0		1	1	3
AU0561	973-2	non-autistic		M		Parent	**56**	1.41 **		0		0			0	
AU0561	974-1	non-autistic		M		Parent	**48**	0.92 **		0		0			0	
AU0561	975-5	PDD	NV	F		Sibling	**44**	0.89 **		0		0		0	0	
AU0574	1219-2	non-autistic		M		Parent	23	0.43		0		1	4		0	
AU0574	1220-3	autistic	V	M		Sibling	27	0.48		1	2	0		0	0	
AU0574	1221-5	PDD	NV-R	F		Sibling	27	0.34		0		0		-1	0	
AU0574	1222-4	autistic	NV	M		Sibling	24	0.27		1	3	0		1	0	
AU0574	1223-1	non-autistic		F		Parent	30	0.4		0		0			0	
AU0575	854-4	autistic	NV	M		Sibling	**50**	0.8 **		0		0		0	0	
AU0575	855-3	autistic	V	M		Sibling	**58**	0.85 **		0		0		1	0	
AU0575	856-1	non-autistic		F		Parent	**60**	0.94 **		0		1	-1		0	
AU0575	857-2	non-autistic		M		Parent	**72**	1.37 **		0		0			0	
AU0430	406-1	non-autistic		F		Parent	**31**	0.4		0		0			0	
AU0430	407-2	non-autistic		M		Parent	26	0.33		0		1	8		0	
AU0430	408-3	autistic	NV	F		Sibling	30	0.19		1	4	-1		0	-1	
AU0430	409-4	autistic	V	M	IT	Sibling	29	0.24		1	4	-1		0	-1	
AU0430	410-5	autistic	NV	M	IT	Sibling	30	0.28		-1		-1		-1	-1	
AU0560	1299-1	non-autistic		F		Parent	**41**	1.3 **								
AU0560	1300-4	autistic	NV	M		Sibling	**31**	0.87 **								
AU0560	1301-2	non-autistic		M		Parent	27	0.58								
AU0560	1302-3	autistic	V-R	M		Sibling	**33**	0.89 **								
AU0560	1303-5	autistic	NV	M		Sibling	**35**	0.58								
AU0720	1334-1	non-autistic		F		Parent		0.29		0		1	5		0	
AU0720	1335-4	autistic	V	M		Sibling		0.18		1	5	1	-1	0	0	
AU0720	1336-3	non-autistic		F		Sibling		0.13		-1		-1		-1	-1	
AU0720	1337-2	non-autistic		M		Parent		0.25		0		1	8		0	
AU0720	1338-5	autistic	NV	F		Sibling		0.16		0		0		1	0	
AU0489	1254-1	non-autistic		F		Parent		0.32								
AU0489	1255-2	non-autistic		M		Parent		0.3								
AU0489	1256-4	autistic	PDD	M		Sibling		0.18								
AU0489	1257-3	autistic	V	M		Sibling		0.2								
AU0371	292-1	non-autistic		F		Parent		0.42		0		0	-1		1	3
AU0371	293-2	non-autistic		M		Parent		0.43		0		1	1		0	
AU0371	294-3	autistic	V	M	IT	Sibling		0.32		0		0	-1	0	0	
AU0371	295-4	autistic	NV	M	IT	Sibling		0.26		0		0	-1	0	0	
AU0459	580-1	non-autistic		M		Parent		0.22								
AU0459	581-2	non-autistic		F		Parent		0.15								
AU0459	582-3	autistic	V	M		Sibling		0.15								
AU0459	583-5	non-autistic		F		Sibling		0.15								
AU0459	584-4	autistic	V	F		Sibling		0.14								
AU0115	402-2	non-autistic		M		Parent		0.54		0		0	-1		0	
AU0115	378-1	non-autistic		F		Parent		0.54		0		1	1		0	
AU0115	379-5	autistic	NV	F		Sibling		0.48		0		1	6	0	1	2
AU0115	380-4	autistic	NV-R	F		Sibling		0.32		-1				0	0	
AU0115	381-3	non-autistic		F		Sibling		0.3		-1		1	7	0	0	
AU0364	996-1	non-autistic		F		Parent		0.75 **								
AU0364	993-2	non-autistic		M		Parent		0.91 **								
AU0364	997-4	autistic	NV-R	M		Sibling		0.49								
AU0364	994-3	autistic	NV	M		Sibling		0.67 **								
AU0364	995-5	autistic	NV-R	F		Sibling		0.31								

MT – Concentration of Metallothionein – bold (levels above 30 μg/ml)
Anti-MT – Anti-Metallothionein IgG
High AB – Significant concentration of anti-MT (significantly higher than mean ± SD control concentration – >.6 OD)
IT – Identicle Twins

Response Type

Select Value		
	No	0
	Yes	1
	N/A	9
	No respo	–1

Resp – Respiratory Disease
Resp Type – Type of Respiratory Disease
1 – Sleep apnea
2 – tracheomalacia, 1996 dx with sleep apnea
3 – stridor? laryngomalacia – scope showed large tonsils – emergent tonsilectomy.
4 – Respiratory Distress
5 – Oxygen supplementation

Aller – Allergies
Aller T – Allergy Type
1 – seasonal allergies
2 – Arthritis
3 – Has both eczyma and asthma
4 – allergies (seasonal and animal) beginning in adulthood
5 – Eczyma
6 – Emphysema
7 – Frequent Sinus Infections
8 – Chronic Bronchitis

HB – Hyperbilirubinemia

GI – Gastrointestinal Disease
GI T – GI Disease Type
1 – 1. constipation in past; 2. diarrhea; 3. colic
2 – 1. Frequent gagging had an endoscopy at age 8; 2. Loose stools
3 – gastroesophageal reflux

Figure 3

Anti-metallothionein IgG in controls. Mean OD ± SD of Controls (age-matched individuals with no family history of autism). Each control serum assayed at least 3 times.

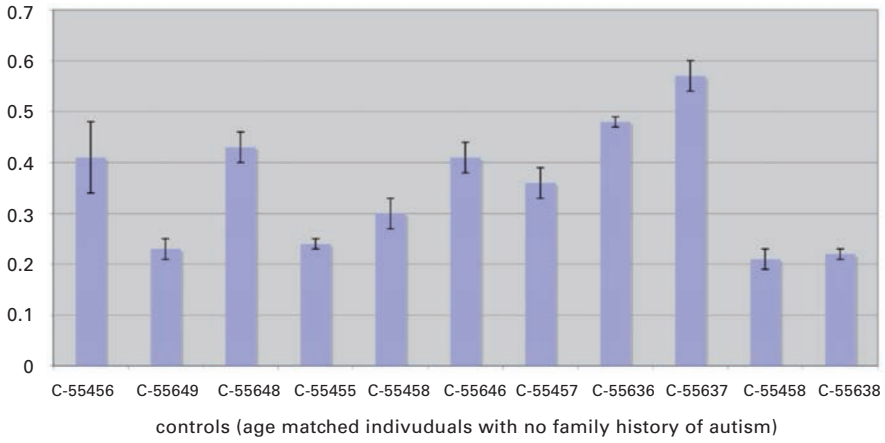
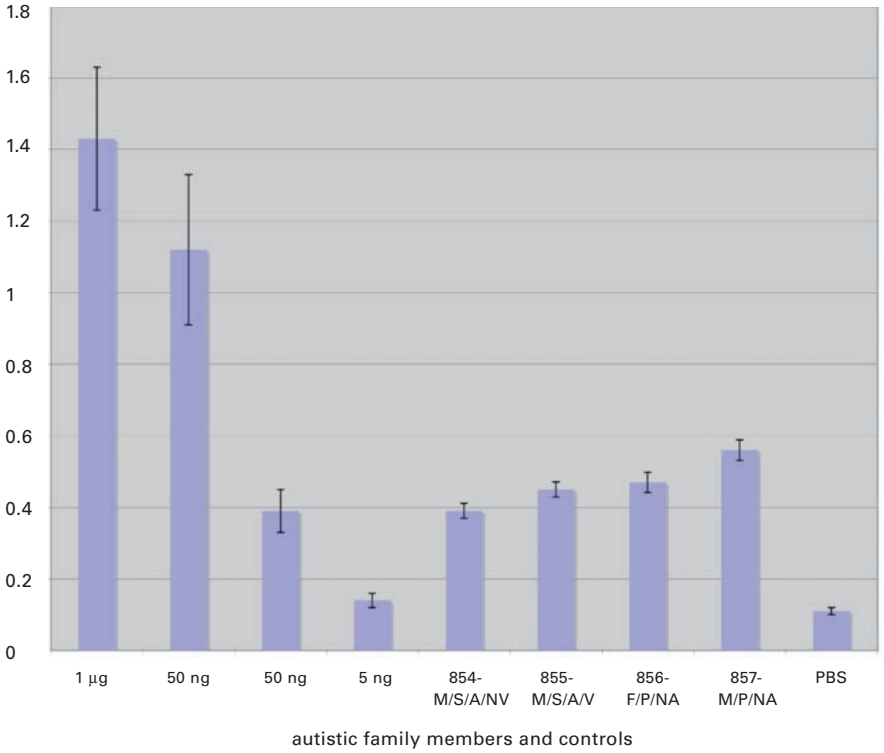


Figure 4

Metallothionein concentration in family members with autistic children. Typical ELISA measuring the concentration of MT in serum from autistic family members compared to controls (+ controls 1-.001 µg/100 µl purified MT; – control PBS).



with no history of autism as controls (from the National Disease Research Interchange). Results are shown in table 1. A typical assay is shown in figure 2. For consistency, all assays were measured when + control OD reached 1.0 ± 0.1.

Twenty-three of the 66 autistic family members (8 parents, 14 autistic children and 1 non-autistic sibling) had auto-antibodies to metallothionein (OD significantly higher than mean of 11 age-matched controls, .35 ± .11; p <.05) (table 1, figure 3).

Three pairs of identical twins with autism were tested for anti-MT IgG. Individuals in each pair had almost identical levels of antibody (table 1).

In all families where anti-metallothionein antibodies were detected (7/14), at least one parent had anti-MT IgG. In five of these families, both of the parents had anti-MT IgG. In the remaining 7 families, none of the members had anti-MT antibodies.

Thirty-nine family members, from 8 of the 14 families with at least one autistic child, were tested for the serum concentration of metallothionein using a direct ELISA. A typical assay is shown in figure 4. Twelve of 17 individuals with high anti-MT IgG also had high MT serum concentration (above 30 µg/ml), whereas only 4 of 22 with no anti-MT IgG had high MT concentration (p <.05) (table 1).

Two of the three pairs of identical twins were tested for MT concentration. Individuals in each pair also had similar concentrations of MT.

There was no significant difference in the concentration of anti-MT IgG when comparing affected children categorised by autistic type (verbal, non-verbal and non-verbal regression) to control individuals (individuals with no family

Figure 5

Anti-MT IgG levels in affected children of different types compared to control groups.

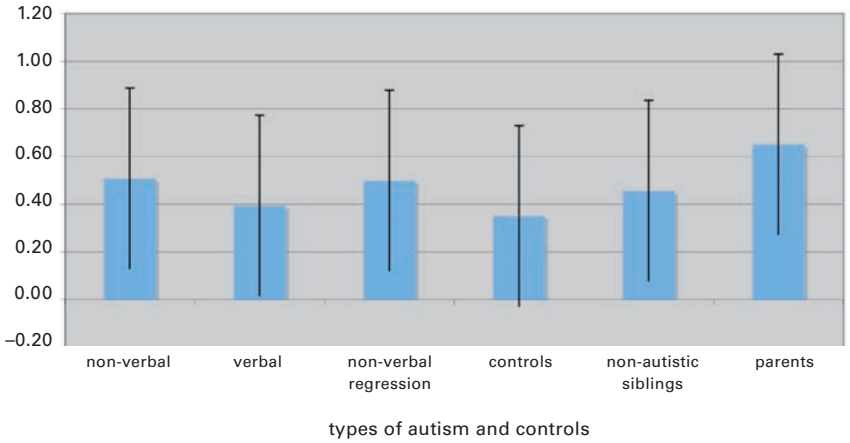
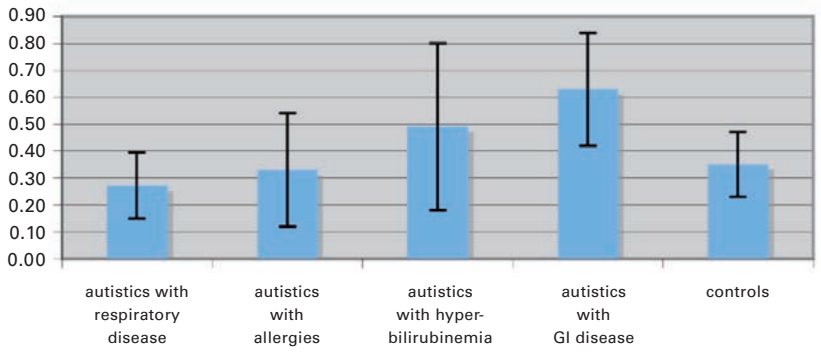


Figure 6

Demographic groups with Anti-MT IgG. Anti-MT levels in affected children of different demographic groups compared to controls (age-matched individuals with no family history of autism). MT contents of normal human serum are typically well below 1 ng/ml.



history of autism, parents and non-autistic siblings) (figure 5). We only assayed one PDD, Aspergers and verbal regression individual and they are not included in this comparison.

Using medical history reports (AGRE), we analysed the phenotypic characterisation of the probands of family members tested in this study (table 1). Thirty-five affected children were tested for anti-MT IgG. Answers to medical history questions were not available for thirteen of these children. Of the 22 remaining, 5 reported respiratory disease (23%). Of these five, none had high levels of anti-MT IgG. We assayed four of these for metallothionein serum concentration. None of these had high levels of metallothionein (figure 6). Although this may suggest that these antibodies may be protective against respiratory disease, the levels of antibody were not significantly different from the amount of antibody in controls.

Only 2 of 22 affected children were reported to have allergies, compared to 9 of 18 parents, and anti-MT IgG levels in these 2 were not significantly different from controls.

Anti-MT antibodies were not significantly different from controls in five affected children with hyperbilirubinaemia and two autistic children with reported GI disease (figure 6).

Discussion

Autoimmune reactions after exposure to heavy metals such as mercury have been causally implicated in autism. As metallothionein (MT) is the primary metal-detoxifying protein in the body, we conducted a study of the MT-1 protein and antibodies to metallothionein (anti-MT IgG) in autistic children, normal children and parents from 14 families, as well as age matched control individuals (no family history of autism).

Metallothioneins (MT), a family of small proteins containing 61–68 amino acids with an unusually high concentration of cysteine, function as intracellular distributors and mediators of heavy metals, including copper and zinc, and heavy metal detoxification.

Data indicates that copper to zinc ratio is abnormally high in individuals with autism [20]. This might be explained by an altered function due to an altered conformation of the protein.

Measurements of MT levels, as well as zinc, have been used to demonstrate zinc deficiency.

MT increases rapidly after zinc supplementation and decreases if the diet is deficient in zinc [21].

Studies indicate that many children with autism have an immune abnormality of some type, including myeloperoxidase deficiency, severe combined immunodeficiency, and IgA deficiencies. Twenty percent of autistics have IgG subclass deficiencies, and deficiencies in complement C4b. Concentrations of IL-12 and interferon gamma are much higher in autistic children than in normal children [22–26]. Autistic individuals are also susceptible to auto-immune disorders [27, 28].

Using an ELISA and western blotting, we searched for serum antibodies to metallothionein-1 in autistic and non-autistic individuals from 14 families, as well as 11 age-matched individuals with no autistic family history. In all families where anti-metallothionein antibodies were detected (7/14), at least one parent had anti-MT IgG. In five of these families, both of the parents had anti-MT IgG. In the remaining 7 families, none of the members had anti-MT antibodies. Also, three sets of identical twins from three families were tested for MT serum concentration and anti-MT IgG. In all cases the concentration of MT and anti-MT IgG was virtually the same in each set. This suggests that the presence of these auto-antibodies is associated with autism, and the production of these antibodies is most likely inherited.

Using a direct ELISA we correlated the presence of anti-MT IgG and concentration of serum metallothionein. In all five families with anti-MT antibodies, the individuals with the highest level of antibodies also had the highest level of metallothionein. In 4 of 5 of these families, the concentration of metallothionein was significantly higher than the 3 families with no antibodies. This suggests that higher levels of metallothionein are associated with anti-MT IgG.

It has previously been reported that anti-MT antibody is present in the circulation of healthy persons, including both normal and autistic children [30, 31].

Singh and Hanson reported in 2006 [29] that serum levels of MT did not significantly differ between normal and autistic children. Furthermore, autistic children harboured normal levels of anti-MT, without any significant difference between normal and autistic children. They suggested 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. Although we found that a significant number of autistic family members (parents and children) had high levels of anti-MT, which corresponded with high levels of MT, the levels of MT and anti-MT did not correlate with autism (parents and non autistic children were just as likely to have high levels), or any type of autism (verbal, non-verbal and non-verbal regression) or demographic group (GI or respiratory disease). This supports the findings of Sing and Hanson.

We could not identify any factors, including immune dysfunction such as allergies, which could explain why only selected families had anti-MT IgG. The presence of anti-MT IgG in some autistic families could be associated with the fact that autistic children are more susceptible to immune dysfunction, including autoimmune disease.

We gratefully acknowledge the resources provided by the Autism Genetic Resource Exchange (AGRE) Consortium and the participating AGRE families. The Autism Genetic Resource Exchange is a program of Cure Autism Now and is supported, in part, by grant MH64547 from the National Institute of Mental Health to Daniel H. Geschwind (PI).

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