Molecular Aspects of Thimerosal-induced Autism

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Summary

The developmental disorder autism has both genetic and environmental origins, and its forty-fold increase during the past two decades reflects an increased role for environmental factors. It has been proposed that increased use of vaccines containing the ethylmercury derivative thimerasol is the major contributing factor. Published research from my laboratory has revealed that thimerosal is an exceptionally potent inhibitor of biochemical pathways that transfer single carbon atoms between molecules. These "methylation" pathways are critically involved in several important functions including the regulation of gene expression and the molecular mechanism of attention. Recent studies from my lab indicate that thimerosal exerts its toxic effect on methylation by interfering with formation of the active form of vitamin B12, also known as cobalamin. Dietary B12 must be converted to methylB12 (methylcobalamin) in order to assist in the transfer of single-carbon methyl groups from the folic acid pathway by the enzyme known as methionine synthase. By reducing methylB12 formation, thimerosal inhibits this enzyme and thereby interferes with methylation events. Autistic children have abnormal plasma levels of methylation-related metabolites and exhibit higher frequencies of genetic mutations that affect this pathway. These genetic risk factors make them less able to detoxify thimerosal and also increase their sensitivity to its mechanism of toxicity. In many cases, autism can be effectively treated by the administration of methylB12 along with other agents that augment methylation capacity. Taken together, these facts indicate that increased exposure to thimerosal has combined with genetic risk factors in a sensitive subpopulation to cause the recent rise in autism.

Outline

1. The Puzzle of Autism

Autism is a pervasive developmental disorder characterized by deficits in language, attention, cognition and learning, frequently accompanied by abnormal

behavior including social isolation, repetitive activity and emotional lability. Severe deficits may be recognized at birth, but a failure to achieve standard milestones during initial years of life remains the primary basis of diagnosis in most cases. While the underlying cause(s) remains obscure for many developmental disorders, metabolic abnormalities (e.g. Lesch-Nyhan Syndrome and adenylsuccinate lyase deficiency) or impaired methylation-dependent gene silencing and/or imprinting (Rett and Fragile-X Syndromes) (1-4) suggest biochemical mechanisms that may be involved. Development disorders can also be caused by exposure to toxins (e.g. ethanol, in fetal alcohol syndrome; heavy metals, in lead poisoning) (5,6), although the precise molecular mechanisms underlying their toxicity are not known. The recent increase in the incidence of autism has led to speculation that environmental exposures including vaccine additives (i.e. aluminum and the ethylmercury-containing preservative thimerosal) might contribute to the triggering of this developmental disorder (7).

Based upon a high concordance in twin studies, genetic factors are thought to play an important role in causing autism. However, it is clear that the recent dramatic rise in autism rates is not caused by a genetic phenomenon. The more likely scenario is that autism is caused by the interaction of genetic risk factors with environmental risk factors and the importance of the environmental factors has increased during the past twenty years. As illustrated in Fig. 1, the "Puzzle of Autism" therefore is the challenge of understanding exactly which genes provide the inborn risk, and which environmental factor(s) is serving as the trigger. The molecular mechanism at the intersection of genetic and environmental factors should be capable of accounting for the observed symptoms of autism, and knowledge of this mechanism should help identify effective treatments for

autism. The findings summarized in this report indicate that impairment in the biochemical pathways that allow for the transfer of single carbon groups (i.e. methylaion) is a major factor contributing to the cause(s) of autism.

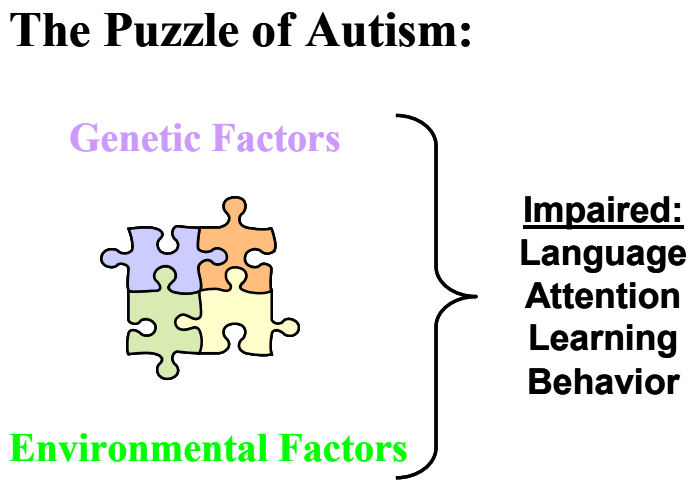

Figure 1: Autism is caused by a combination of predisposing genetic factors and environmental factors that synergize with each other to cause the symptoms that are typical of this developmental disorder.

2. Physiological and Biochemical Roles of Methylation

Methylation is the process by which a single carbon atom is transferred from a methyl donor to another molecule, commonly resulting in a change in the functionality of the recipient molecule. This seemingly mundane biochemical event is vital to life and to the normal capacities of developed organisms, including man. Perhaps the most important example of methylation is the epigenetic regulation of gene expression by DNA methylation. When DNA is methylated, gene expression is suppressed, and at any one time only a portion of genes are “on” with the others being turned “off”. Since all cells possess the same DNA, differences between cell types (e.g. neurons *vs.* heart muscle *vs.* liver cells) are due to specific patterns of DNA methylation that characterize each type. Development begins with undifferentiated cells (i.e. stem cells) that gradually assume the characteristics of their final destiny as guided by sequential shifts in their

DNA methylation. Based upon this perspective, it is easy to see how abnormal methylation could alter the pathway of normal development and could contribute to neurodevelopmental disorders such as autism. Indeed, abnormal DNA methylation has previously been implicated as an important causative factor in Rett and Fragile-X syndromes (3,4)

As illustrated in Fig. 2, the major methyl donor in biological reactions is S-adenosylmethionine (SAM), an activated form of the essential, sulfur-containing, amino acid methionine. After donating its methyl group, the residual portion of SAM, S-adenosylhomocysteine (SAH), serves as a regulator of methylation by competing with SAM and inhibiting its methyl donation. The concentration ratio of [SAM]/[SAH] therefore reflects the potential for methylation, and any increase in [SAH] or decrease in [SAM] will lower methylation. As described below, children with autism have low levels of SAM and elevated levels of SAH, indicating an impaired potential for methylation. Methylation of neurotransmitters such as dopamine and serotonin terminates their signaling activity, which may also play a role in autism.

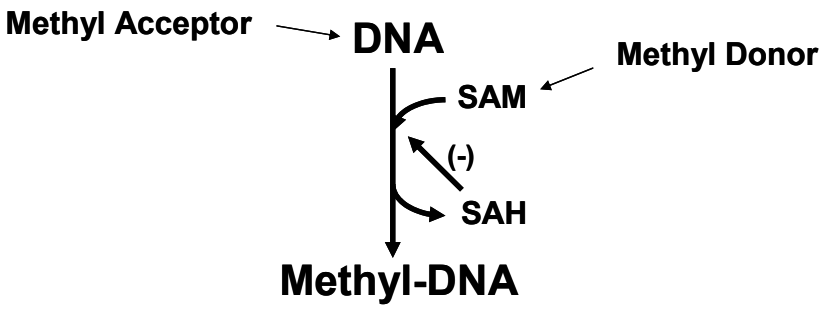

Figure 2: DNA methylation is carried with S-adenosylmethionine (SAM) serving as the methyl donor. The resulting S-adenosylhomocysteine (SAH) inhibits methylation by competing with SAM.

Availability of the methyl donor SAM is critical for methylation. SAM is formed by addition of an adenosyl group from the high energy molecule ATP to methionine, as a

part of the methionine cycle illustrated in Fig. 3. After methyl donation the adenosyl group is removed from SAH, in a reversible reaction yielding homocysteine (HCY) and adenosine. Any unusual build-up of adenosine can shift this reaction backwards toward SAH formation, while lowering HCY levels. As described below, this occurs in many children with autism. Activity of the vitamin B12-dependent enzyme methionine synthase converts HCY back to methionine, using a methyl group from the folate pathway.

METHIONINE SYNTHASE AND THE METHIONINE CYCLE

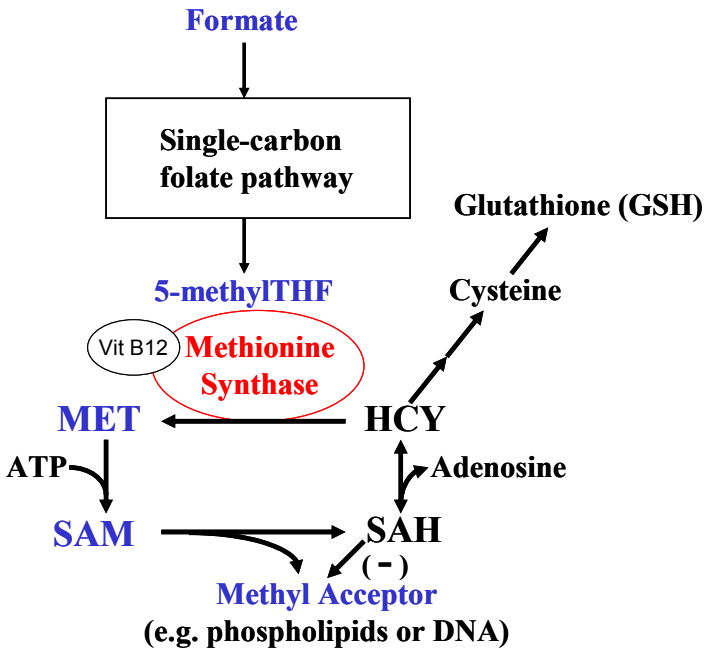

Figure 3: The four-step methionine cycle involves activation of methionine (MET) by ATP-dependent adenosylation, methyl donation by SAM, reversible dissociation of SAH, and remethylation of homocysteine (HCY) to MET by the vitamin B12-dependent enzyme methionine synthase, using methylfolate (5-methylTHF) as the methyl donor. HCY can alternatively be converted to cysteine and glutathione.

The methionine cycle is also involved in the ability of the neurotransmitter dopamine to stimulate methylation of phospholipids in the neuronal membrane. This

unique process was only discovered several years ago and its precise function remains unclear at this time. However, dopamine-stimulated phospholipid methylation (PLM) appears to be involved in the molecular origins of attention. Genetic variations in the D4 subtype of dopamine receptor that carries out PLM have been linked to attention-deficit hyperactivity disorder (ADHD) (8), and the ADHD-linked variant form is weak in its ability to carry out methylation (9). Impaired attention is a cardinal symptom of autism, and it is possible that this reflects reduced activity of dopamine-stimulated PLM. During dopamine-stimulated PLM, a methionine that is an integral part of the D4 receptor protein is converted to SAM, then SAH, then HCY and back to methionine again, as in the methionine cycle of Fig. 3. Thus enzymes in the methionine cycle, such as methionine synthase, actually have two substrates, one being a small individual amino acid, and the other being the large D4 dopamine receptor protein.

3. Activity of Methionine Synthase

Methionine synthase is situated at the intersection of the single-carbon folate pathway and the methionine cycle (Fig. 3), and is therefore well-positioned to regulate methylation. Its activity serves to maintain a low level of HCY, limiting its backward conversion to SAH and thereby promoting methylation. In a recently published study (10), we showed that methionine synthase activity in cultured human neuronal cells is substantially stimulated by both dopamine and insulin-like growth factor-1 (IGF-1) (Table 1). IGF-1 mediates many of the effects of growth hormone and is a key regulator of development, as well promoting neuronal myelination.

The mechanism of methionine synthase activation involves an intracellular signaling pathway, the PI3-kinase pathway, commonly activated by many different

cellular growth factors, including those that promote cellular differentiation and development. In subsequent investigations we found that methionine synthase activity in neuronal cells is absolutely dependent upon the ability of this signaling pathway to promote the formation of the biologically active form of vitamin B12 (i.e. methylB12 or methylcobalamin). It is pathway that is inhibited by thimerosal.

METHIONINE SYNTHASE ACTIVITY[1]

Treatment	pmol/min/mg
Basal	28.5 ± 4.3
IGF-1 (10 nM; 30 min)	62.2 ± 2.8
Wortmannin (100 nM; 60 min)	not detectable
IGF-1/Wort.	not detectable
Dopamine (10 µM; 30 min)	76.0 ± 3.7
Dopamine/Wort.	0.9 ± 1.2
Dopamine/IGF-1	132.1 ± 7.7
Ethanol (0.1%; 60 min)	not detectable
IGF-1/Ethanol	1.0 ± 1.3
Dopamine/Ethanol	not detectable
$HgCl_2$ (1 µM; 60 min)	not detectable
IGF-1/$HgCl_2$	not detectable
Dopamine/$HgCl_2$	not detectable
$PbNO_3$ (1 µM; 60 min)	2.6 ± 1.5
IGF-1/$PbNO_3$	37.9 ± 2.9
Dopamine/$PbNO_3$	26.3 ± 3.1
Thimerosal (10 nM; 60 min)	not detectable
IGF-1/Thimerosal	not detectable
Dopamine/Thimerosal	not detectable

Table 1: Effects of various agents on methionine synthase activity in neuronal cells. IGF-1 and dopamine stimulate activity, while the PI3-kinase inhibitor wortmannin, ethanol, mercury ($HgCl_2$), lead ($PbNO_3$) and thimerosal inhibit activity.

In the diet we take in vitamin B12 as its hydroxyl derivative, hydroxycobalamin, which must be subsequently converted to methylcobalamin before it can function. Dietary vitamin supplements provide cyanocobalamin, which again must be converted to methylcobalamin. Conversion to methylcobalamin can occur either directly in the

enzyme methionine synthase itself, or via the pathway outlined in Fig. 4. As illustrated, methylcobalamin synthesis requires glutathione (GSH) and SAM, and levels of each of these metabolites are reduced in autistic children (see below). Although additional studies are needed to clarify details, growth factors apparently augment synthesis of the intermediate glutathionylcobalamin, which is subsequently converted to methylcobalamin. The resultant higher level of methylcobalamin increases methionine synthase activity, lowering HCY and SAH levels and increasing methylation. In support of this mechanism, our published study showed that IGF-1 and dopamine increase the methylation of both DNA and membrane phospholipids in conjunction with their activation of methionine synthase.

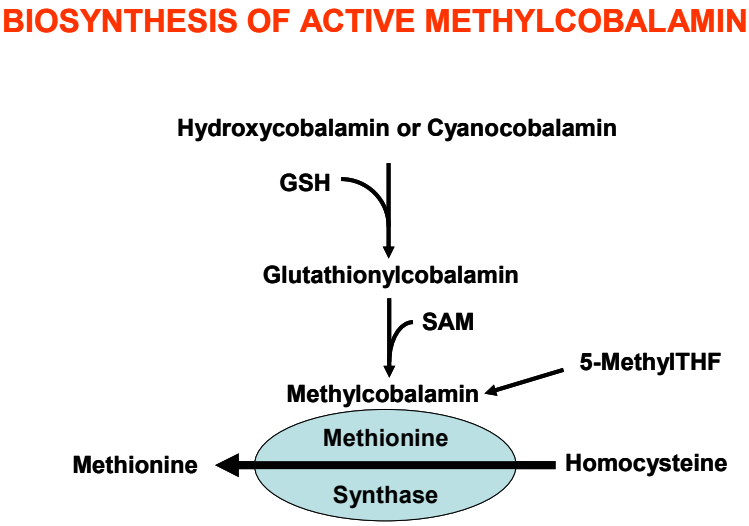

Figure 4: Dietary or multivitamin forms of vitamin B12 (cobalamin) must be converted to the active methylcobalamin form via a two-step process requiring glutathione (GSH) and SAM.

As illustrated in Fig. 5 (left), methionine synthase normally contains four domains: 1. A cobalamin-containing catalytic domain. 2. A methylfolate-binding domain. 3. A HCY-binding domain. 4. A SAM-binding domain. During the catalytic cycle, folate and HCY domains alternatively interact with the cobalt ion in cobalamin, which

alternates between Cob(I) and methylated Cob(III) states. Cob(I) is, however, extremely unstable, and occasionally it oxidizes to the Cob(II) state, interrupting folate-dependent HCY methylation. Oxidation is especially likely when levels of methylfolate are low and the Cob(I) state has to wait too long to receive a methyl group. Under this circumstance, the SAM-binding domain, when present, carries out a reductive methylation of Cob(II), with the auxiliary assistance of methionine synthase reductase. Thus the SAM-binding domain rescues oxidized cobalamin, allowing methionine synthase activity to resume. Alternatively, oxidized Cob(II) can be replaced with a new molecule of methylcobalamin to restart the enzyme. Thus oxidized cobalamin can either be repaired or replaced, but replacement places a high demand on methylcobalamin synthesis.

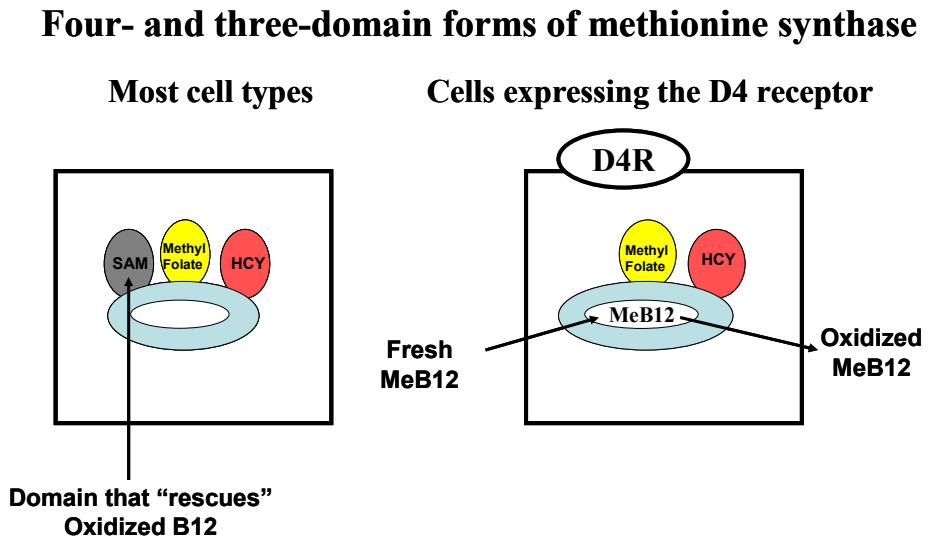

Figure 5: Methionine synthase can exist in both four-domain and three-domain forms. In the three-domain form, the SAM-binding domain that rescues oxidized Cob(II) is missing. In cells containing only the three-domain form, oxidized B12 must be replaced with methylB12 to resume enzyme activity.

In very recent and as yet unpublished studies, we have found evidence indicating that methionine synthase also exists with only three domains, with the SAM-binding domain being absent (Fig. 5, right). This form of the enzyme lacks the ability to rescue oxidized cobalamin, and therefore is highly dependent upon the availability of

methylcobalamin to sustain activity. As such, this form of the enzyme is subject to regulation by growth factors and the PI3-kinase signaling pathway, since they control the level of methylcobalamin synthesis. The particular human neuronal cell line we utilized contained only the three-domain enzyme. As a consequence, its methionine synthase activity and its methylation activity were tightly and completely under the control of the growth factors signaling pathway.

What would be the advantage to a cell of having a form of methionine synthase that could not repair its oxidized cobalamin co-factor? While we do not conclusively know the answer to this question, we hypothesize that the absence of the SAM-binding domain may improve the ability of the enzyme to utilize the D4 dopamine receptor as a substrate, since it is a larger, more bulky substrate than HCY, and the three-domain form is more prominent in cells expressing the D4 receptor. If correct, this would imply that the synthesis of methylcobalamin is of particular importance in those neuronal cells that express the D4 receptor. Moreover, toxic agents that impair methylcobalamin synthesis would particularly affect the methylation function of D4 receptors, and would therefore cause impaired attention.

4. Effects of Thimerosal and Heavy Metals

As described in our published study, a number of neurodevelopmental toxins share the ability to potently inhibit methionine synthase activity and methylation. These include ethanol, which causes fetal alcohol syndrome, heavy metals such as lead, which causes lead poisoning, as well as mercury and thimerosal. Fig. 6 illustrates the dose-dependent inhibition of phospholipid methylation (PLM) by lead and mercury. It is of particular note that concentrations of lead that reduce cognitive function (IQ) (6)

significantly inhibit PLM. Thimerosal, which releases ethylmercury, was more than 100-fold more potent than inorganic mercury at inhibiting methylation (Fig. 7). Ten days after vaccination with a thimerosal-containing vaccine, the concentration of ethylmercury in blood is reported to be approximately 8 nM (11). In our study, this concentration produced greater than 50% inhibition of methylation. Assuming that these blood levels are also present in the brain, one could reasonably expect that vaccine-derived doses of thimerosal inhibit methylation in the brain.

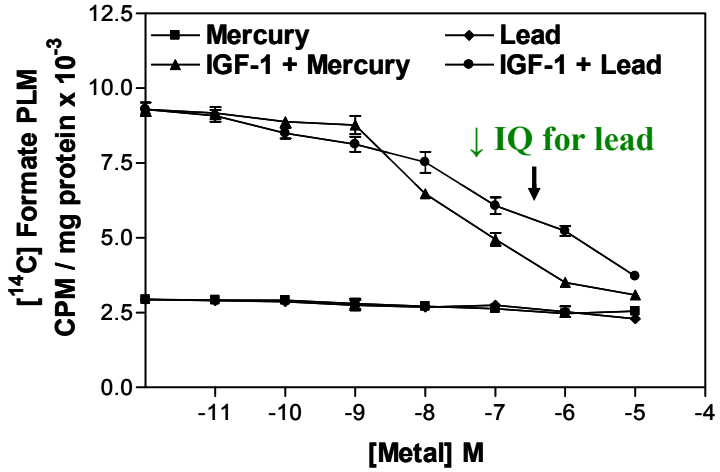

Figure 6: Mercury and lead potently inhibit the ability of IGF-1 to stimulate phospholipid methylation in human neuroblastoma cells.

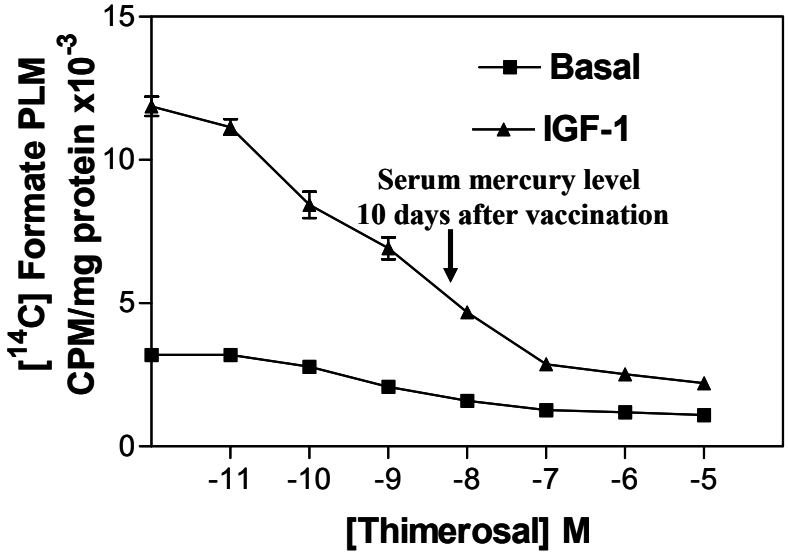

Figure 7: Thimerosal potently inhibits IGF-1-induced phospholipid methylation. Blood levels found in children ten days after vaccination produced approximately 50% inhibition.

Thimerosal, ethanol, mercury and lead also inhibited methionine synthase activity. As shown in Table 1, enzyme activity (i.e. methylation of HCY) was undetectable after a 30 min pretreatment with a thimerosal concentration close to the blood level found after vaccination (10 nM). Thus inhibition of methionine synthase accounts for the inhibitory effect of thimerosal on methylation. The toxic effect of thimerosal was also evident simply by observing the shape of cells, which changed from their usual spindle shape to a condensed, round shape (Fig. 8).

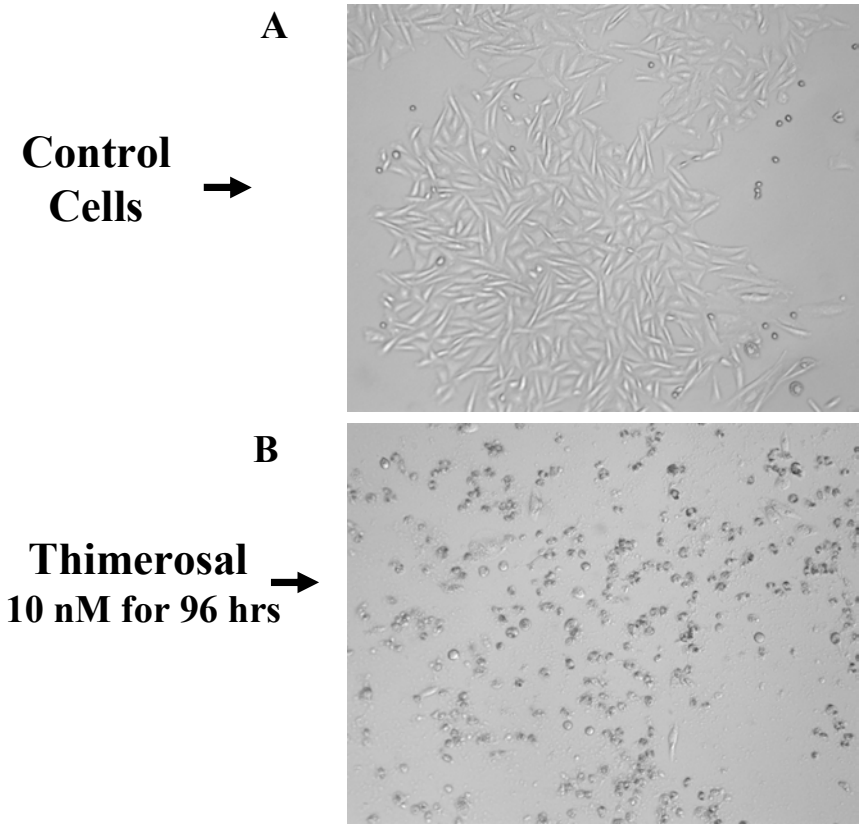

Figure 8: Thimerosal induces a dramatic change in the morphology of human neuroblastoma cells.

We further investigated the mechanism by which thimerosal inhibits methionine synthase. As shown in Fig. 9 (bottom), when enzyme activity was measured in the presence of either hydroxycobalamin or cyanocobalamin, thimerosal caused almost complete inhibition, however in the presence of methylcobalamin, thimerosal caused no

inhibition. Furthermore, when activity was measured in the presence of glutathionylcobalamin and SAM, thimerosal inhibition was again absent, although when SAM was not added, inhibition was observed. This pattern indicates that thimerosal inhibits the availability of glutathionylcobalamin, and that this action is responsible for its inhibition of methionine synthase and methylation.

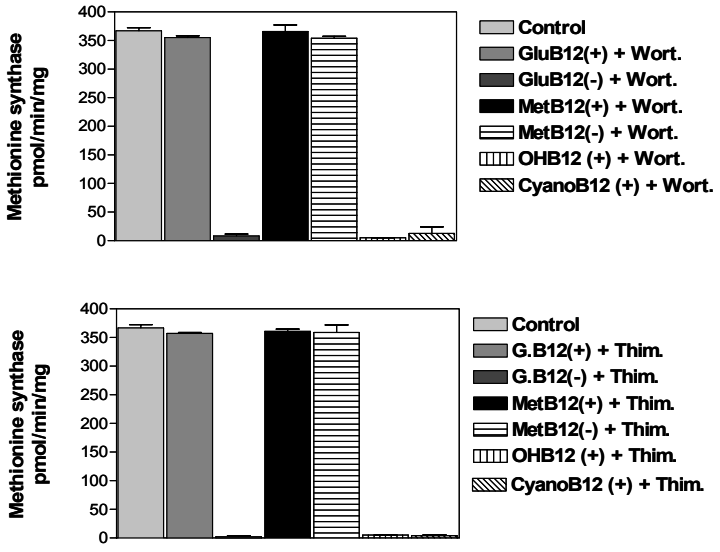

Figure 9: The PI3-kinase inhibitor wortmannin and thimerosal eliminate the ability of hydroxo- and cyanocobalamin to support methionine synthase activity. The presence of SAM is indicated by (+).

We also examined the ability of different cobalamins to support methionine synthase activity after inhibition of PI3-kinase. Treatment with the selective PI3-kinase inhibitor wortmannin caused a pattern of absolute dependence on methylcobalamin or its synthesis (gluthionylcobalamin + SAM) that was identical to the effect of thimerosal (Fig. 9, top). Since thimerosal and wortmannin produce identical effects, this data strongly suggests that thimerosal acts by inhibiting the PI3-kinase signaling pathway. **This is the likely mechanism by which thimerosal causes autism, and may also be the molecular basis for its toxic effect on bacteria, fungi that makes it an effective preservative.**

5. Autism-associated Metabolic and Genetic Abnormalities

Metabolic and genetic studies of autistic subjects provide a more complete view of how thimerosal, as an environmental insult, causes autism. Some of the most compelling information has only recently been obtained, and we are all indebted to the ongoing work of Jill James, Jeff Bradstreet, Marvin Boris, Alan Goldblatt, Ted Page, Gene Stubbs and others.

As described in a recent study by Dr. Jill James (12), the concentration of each of the individual metabolites in the methionine cycle and the trans-sulfuration pathway leading to glutathione synthesis is significantly abnormal in autistic children as compared to normal controls (Table 2). Notably, methionine and SAM levels are low, consistent with lower activity of methionine synthase. While a low HCY level might not be expected, the elevated levels of both SAH adenosine indicate that HCY is being drawn backwards toward SAH via the reversible activity of the enzyme SAH hydrolase. Thus an elevated level of adenosine restricts the availability of HCY for both methionine (and SAM) synthesis and for the formation of cysteine and glutathione.

	Control Children n=33	Autistic Children n=20	p value
Methionine (μmol/L)	30.6 ± 6.5	19.3 ± 9.7	0.001
SAM (nmol/L)	90.0 ± 16.2	75.8 ± 16.2	0.01
SAH (nmol/L)	20.1 ± 4.3	26.1 ± 5.4	0.001
Homocysteine (μmol/L)	6.3 ± 1.2	5.4 ± 0.9	0.01
Adenosine (μmol/L)	0.28 ± 0.16	0.39 ± 0.19	0.05
Cysteine (μmol/L)	210 ± 18.5	163 ± 14.6	0.001
Total glutathione (μmol/L)	7.9 ± 1.8	4.1 ± 0.5	0.001
Oxidized Glutathione (nmol/L)	0.3 ± 0.1	0.55 ± 0.2	0.001
GSH/GSSG Ratio	25.5 ± 8.9	8.6 ± 3.5	0.001

Table 2: Metabolites in the methionine cycle and transsulfuration pathway are abnormal in autism (data from Dr. Jill James).

The 20% lower levels of cysteine and 54% lower levels of glutathione in autistic children will adversely affect their ability to detoxify and excrete heavy metals and thimerosal. These two compounds directly bind inorganic and organic mercury and help direct them to the kidneys for excretion. As a result, these toxic materials will reach a higher free concentration in the bloodstream of autistic children, will have an increased potential for transfer to tissue compartments such as the brain, and will remain in the body for a significantly longer period of time, as compared to their counterparts who have normal levels of cysteine and glutathione. These differences begin to define the subpopulation of children who are more vulnerable to thimerosal and heavy metal exposure.

Earlier metabolic and genetic studies provide clues to the cause of the increased adenosine level in autism. Page and co-workers found 8 to10-fold higher activity of the enzyme that makes adenosine (5'-nucleotidase) in subgroup of children (13), while Stubbs and co-workers found that the enzyme that degrades adenosine (adenosine deaminase) has lower activity in autistic subjects (14). Genetic studies have also shown that a polymorphism in the adenosine deaminase that weakens the enzyme is more common among autistic subjects (15). Impairment of adenosine deaminase, may result from dysfunctional interactions with its binding partner, enzyme dipeptidyl peptidase IV. As illustrated in Fig. 10, these metabolic defects can combine with thimerosal exposure and other genetic risk factors to inhibit methylation and cause autism.

There is recent evidence that polymorphisms in genes for methionine synthase and closely-related enzymes are another source of risk for autism. For example, there are two well-characterized disabling polymorphisms in the methylenetetrahydrofolate

reductase (MTHFR) gene, the enzyme that makes methylfolate available to methionine synthase, and these polymorphisms are more common in autism (16). MTHFR polymorphisms reduce methylfolate levels, which slows the methylation of Cob(I) and increases the probability that it will oxidize to Cob (II). As a consequence, MTHFR polymorphisms increase methylcobalamin demand for the three-domain form of methionine synthase. A disabling polymorphism in methionine synthase, in a location that can affect the proportion of three- *vs.* four-domain enzyme forms, is reported to be six-fold more prevalent in autistic children (17). Finally, a polymorphism in the enzyme methionine synthase reductase, which assists in the rescue of cobalamin, may also be more frequent in autism (18). While other polymorphisms remain to be discovered, these examples serve as examples of genetic risks that characterize autistic children, making them more sensitive to the toxic effect of thimerosal and more prone to develop autism.

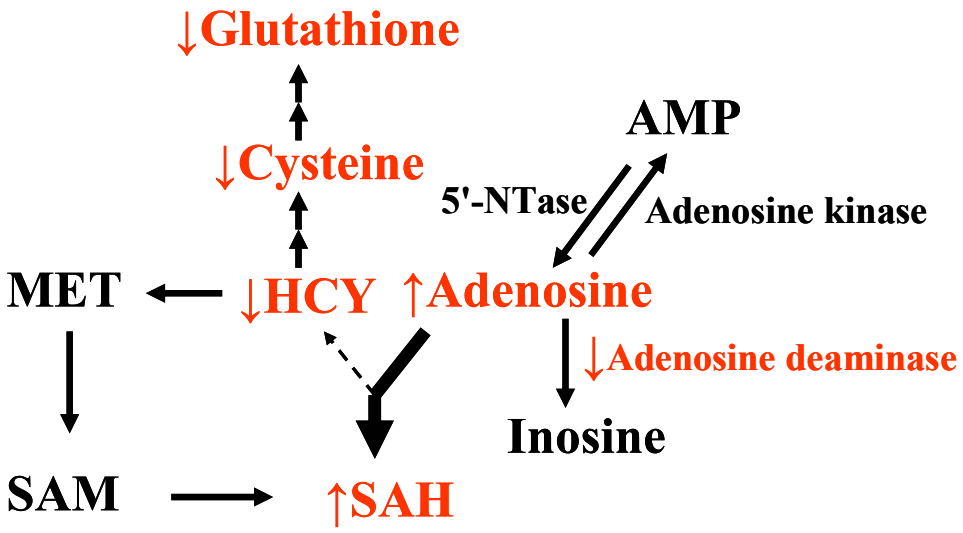

Figure 10: Decreased activity of adenosine deaminase or increased activity of 5'-nucleotidase (5'-NTase) can increase adenosine levels, resulting in lower levels of HCY, cysteine and glutathione.

6. Methylation-related Treatments for Autism

If impaired methylation is important in causing autism, metabolic interventions that augment methylation should be effective treatments. More specifically, if thimerosal's inhibition of methylcobalamin synthesis is important in causing autism, then the administration of methylcobalamin should significantly improve autism. Indeed, this has proved to be the case. As first reported by Dr. James Neubrander (19), injections of methylcobalamin, given once every three days, has brought about significant improvement in approximately 80% of children with autism. While the degree of improvement varies, a significant number of children have improved to the point that they are no longer considered to be "on the autism spectrum". Areas of particular improvement include language, attention and social skills, which are hallmark symptoms of autism. Within the next few months, the M.I.N.D. Institute at the University of California at Davis School of Medicine is slated to carry out a controlled study of methylcobalamin effectiveness in autism.

Other methylation-promoting treatments are also proving helpful in autism. In the metabolic study carried out by Dr. Jill James and colleagues (12), autistic subjects were treated with folinic acid (leucovorin), a folic acid derivative that augments levels of 5-methylTHF, along with betaine (trimethylglycine), which feeds methyl groups to the folate pathway. These two agents normalized most of the abnormal metabolites listed in Table 2, and this was accompanied by clinical improvement in autism symptoms. Subsequent addition of methylcobalamin to this regimen brought about further improvement.

While encouraging, these metabolic interventions do not help many autistic children, and there is a need for additional treatment approaches. Moreover, improving methylation capacity is only one component of the multi-dimensional approach to treating autism. Other elements such as a gluten-free/casein-free diet, chelation of heavy metals and intensive behavioral therapy are also important. Additional metabolic interventions, particularly interventions directed at normalizing adenosine metabolism may prove fruitful. Clearly further research is needed, building upon the framework of knowledge about how genetic and environmental factors can synergize to cause autism.

7. Conclusions

Autism is a neurological disorder caused by dysfunctional metabolic control over methylation reactions, and thimerosal appears to be a precipitating causative factor in many cases. The methionione cycle and the trans-sulfuration pathway leading to cysteine and glutathione synthesis are abnormal in autism. Genetic polymorphisms, present in only a small subpopulation, represent risk factors for autism. As illustrated in Fig. 11, some of these genetic factors impair detoxification and clearance of heavy metals, including thimerosal, and also impair the capacity for methylation. Delayed clearance of thimerosal further impairs methylation, including both DNA methylation and dopamine-stimulated phospholipid methylation, adversely affecting growth factor-directed development and the capacity for attention, respectively. Autism can be treated, and some of the most effective treatments, such as methylcobalamin, act by improving methylation. This encouraging therapeutic development reinforces the conclusion that thimerosal does indeed cause autism, and it does this by interfering with methylcobalamin synthesis. This

molecular understanding should lead to new and improved treatments for autism and should provide a scientifically sound basis for the removal of thimerosal from all vaccines.

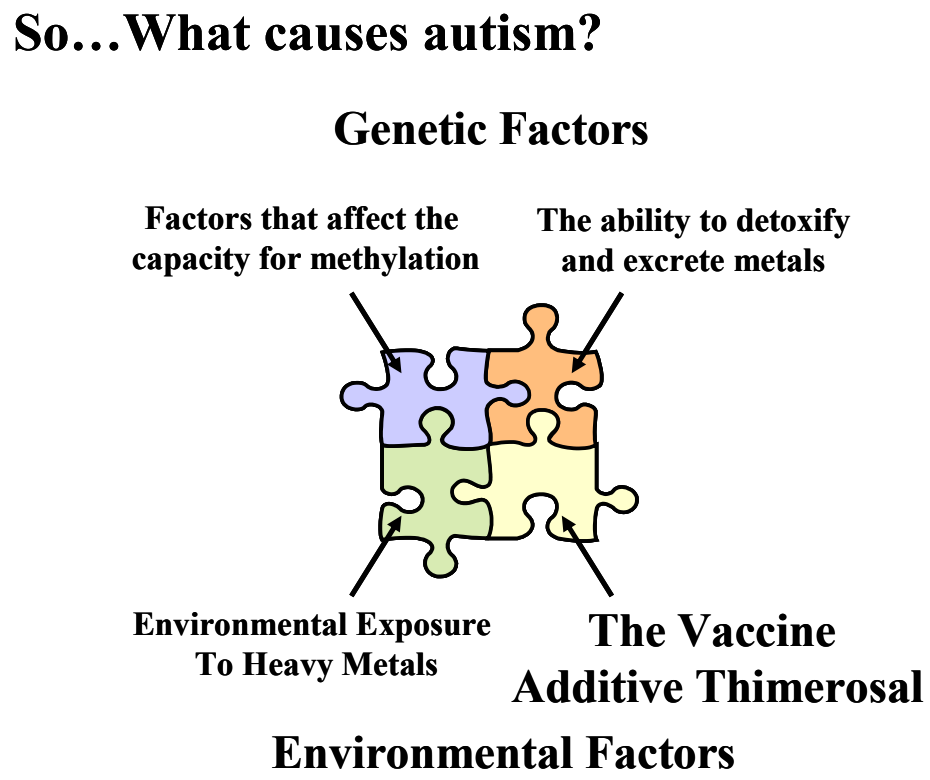

Figure 11: Genetic and environmental factors combine to cause autism.

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