Respondent's Exhibit S

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Comments on the hypothesis that there is a relationship between thimerosal and autism linked in a causal way by oxidative stress and altered sulfur metabolism.

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In the following, I provide my opinions concerning the hypothesis that there is a causal relationship between exposure to thimerosal in vaccines and autism, which involves pathologic alterations in sulfur metabolism. I begin my report by reviewing sulfur metabolism as a background and then respond to the hypotheses outlined in an expert report entitled: "The Relationship Between Thimerosal and Autism" authored by Richard C. Deth, Ph.D. My opinions are based upon my knowledge of glutathione, oxidative stress, and toxicologic mechanisms obtained during the past 30 years as a scientific investigator studying these areas, and upon my review of Dr. Deth's report and related research articles.

Qualifications

Research interests: Biochemical mechanisms of disease; Glutathione metabolism and function; Oxidative stress; Biochemical toxicology; Diet and disease prevention.

Education: B.S. degree in Chemistry and Biochemistry (Univ. of Illinois, Urbana); Ph.D. in Medical Biochemistry (Univ. of Oregon Health Science Center, Portland; Dissertation: Biochemistry of Hypoxia), Post-doctoral training in Nutritional Biochemistry (Cornell Univ., Ithaca, NY) and Biochemical Toxicology (Karolinska Institute, Stockholm).

Employment: Currently, Professor of Medicine (Pulmonary Division) and Director of the Clinical Biomarkers Laboratory, Emory Univ. Previously: Dept. of Biochemistry (1979-85, Asst. Prof.; 1985-91, Assoc. Prof.; 1991-2003, Prof.), Emory Univ.

Publications: Over 300 research papers, including 198 peer-reviewed publications and 112 invited reviews and book chapters.

Memberships and Honors: Society for Toxicology, Society for Free Radical Biology and Chemistry, American Society for Nutritional Sciences. Previously: American Chemical Society, American Society for Biological Chemistry, American Physiological Society, American Research in Vision and Ophthalmology. President, Hypoxia Specialty Section, Am. Physiol. Soc.; President, Mechanisms Specialty Section, Society of Toxicology. NIH Grant Review Panels; Chair, Alcohol and Toxicology I Study Section; Albert E. Levy Award (Emory University’s highest research award).

Overview

- Glutathione is available in abundance. In addition to glutathione, other thiol and disulfide forms of proteins are available, which provides stability that makes the biological system resistant to acute perturbations and adaptive to chronic perturbations.

- It is inappropriate to assume that 1- and 2- carbon compounds (methyl- and ethyl-compounds) share toxic risks.

- Changes in sulfur metabolism that may occur following thimerosal exposure cannot be said to be an adverse effect and may instead be a beneficial response.

- In cysteine metabolism, glutathione is "downstream" from methionine, and there is little if any evidence that perturbations downstream in cysteine-related metabolism have any effects upon its upstream components.

- If autism is caused by alterations in glutathione metabolism, then autism would be expected to be caused by hundreds of other factors that also affect glutathione metabolism.

- Cell studies are difficult to extrapolate to in vivo toxicity and are not reliable as a basis to determine causality.

Sulfur in Biological Systems

Sulfur is the 5th most abundant element in biological systems, with the reduced thiol form being especially important as a component of protein, cofactors required for protein function, and defense systems which protect against toxicity. Considerable scientific information is available concerning the functions, metabolism, and interactions of sulfur-containing chemicals in biological systems. The following summarizes some key aspects of sulfur utilization and metabolism in biological systems, with specific reference to glutathione, an abundant molecule which has a central role in sulfur metabolism.

Abundance: The total amount of sulfur in the human body is about 0.25% of the body total weight, or between 2 and 2.5 g/kg. This is equivalent to about 62 mmol/kg (i.e., 2 g x 1 mol/32 g). Sulfur is a component of most proteins. A large fraction of this sulfur is present in the reduced, thiol form and another large fraction is present as an oxidized, disulfide form. Sulfur is also present in a highly oxidized form (sulfate) in many complex sugars which are bound to proteins. Sulfur is a component of important cofactors (Coenzyme A and lipoic acid), which are required for energy metabolism, carbohydrate metabolism, and fat metabolism. Thus, all aspects of living systems are either directly or indirectly dependent upon sulfur metabolism.

Chemistry: The sulfur atom exists in a range of stable and unstable structures which are essential to catalyze the diversity of chemical reactions needed for living systems. These structures include a series of oxidation states which range from the most reduced (H_2S) to the most oxidized (SO_4^{-2}).

H_2S	R-S-H	R-S-S-R	$R\text{-}SO_3^{-1}$	$R\text{-}OSO_3$	SO_4^{-2}
Most Reduced	Thiol (Glutathione)	Disulfide	Sulfonate (Taurine)	Bound sulfate	Sulfate Most oxidized

The thiol form is a common functional form in proteins in which the sulfur is bonded to a carbon in a structure, designated "R", and to hydrogen (H) in the structure R-SH. This is a major form of the sulfur in the human body and is relevant to metal ion binding and oxidative stress. The total thiol in humans is about 20 mmol/kg body weight. In terms of the common amino acid cysteine, this would be equivalent to 2,400 mg/kg. In binding to metal ions, including copper, zinc, mercury, and lead, the H is replaced by the metal.

Structure: The range of stable chemical structures of sulfur atoms is used as an important component in the structure of biologic molecules and their assembly to create living cells and organisms. Importantly, the abundance of both thiol and disulfide forms in proteins provides a stability which makes biologic systems resistant to acute perturbations and adaptive to chronic perturbations.

Regulation: Sulfur atoms present in the reduced, thiol form can reversibly lose electrons to become disulfide forms. In this process, two molecules can be converted to one molecule and large molecules can change their shape. This property is used widely to control biologic processes.

Detoxification: Sulfur-containing biochemicals provide important reagents for protection against both endogenous and exogenously derived chemicals which can injure or kill cells and organisms. Three central, interrelated processes are involved, namely, reduction of oxidizing chemicals, detoxification of reactive electrophiles, and binding to metal ions.

Toxicity: The sulfur-containing detoxification systems are overwhelmed by high exposures to oxidizing chemicals, reactive electrophiles, and metal ions. Oxidizing chemicals such as hydrogen peroxide and hypochlorous acid (bleach) function as disinfectants by overwhelming the protective sulfur systems and destroying other biological molecules. This process is non-specific, damaging hundreds to thousands of different sulfur atoms wherever the toxic oxidative chemical is present. Acrolein, a toxic chemical in smoke, and NAPQI, the chemical causing toxicity from acetaminophen overdose, are chemicals which react with sulfur atoms of a large number of different protein targets. Certain metals, including but not limited to iron, copper, zinc, mercury, cadmium, arsenic, and lead, also bind to sulfur atoms in a large number of different proteins. Some of these interactions are highly specific and are required for biological function. Each of the metal ions, when present in excess, causes toxicity by disrupting the normal function of sulfur-containing biological molecules. Which

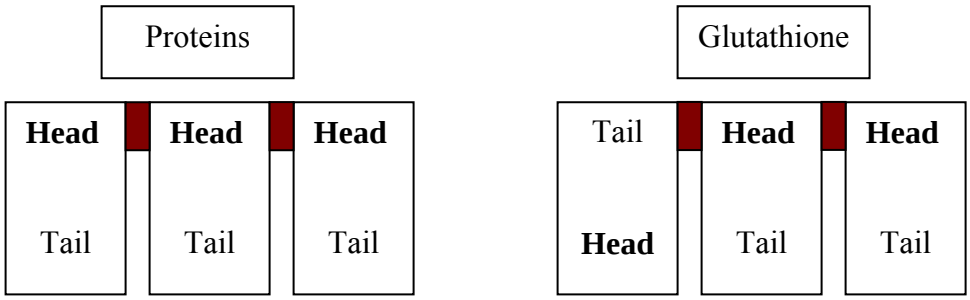
of the many sulfur-containing compounds is the target of the toxic effects of mercury has not been established.

Metabolism and Functions of Glutathione

Glutathione is an abundant sulfur-containing chemical in most organisms. Glutathione has been extensively studied in humans, animals, cell culture, and cell tissue extracts. The information obtained from these studies has established two central biological functions of glutathione: to maintain homeostasis of sulfur metabolism and to protect against the three general types of toxicity described above. The structure and chemical properties of glutathione are well-suited to perform these two central functions.

Structure: Glutathione is composed of three amino acids: glutamate, cysteine, and glycine. These amino acids are among the common 20 amino acids used in protein synthesis. None of the common amino acids in glutathione is considered nutritionally essential in humans, because each can be synthesized from other dietary precursors. Each of the amino acids has other functions in biological systems, with glutamate functioning as an excitatory amino acid and cysteine being a precursor for taurine, an important chemical for regulation of cell volume. Both glutamate and cysteine are toxic to neurons when present in excess. Cysteine causes oxidative stress at high concentrations and this characteristic necessitates maintenance of cysteine at a low concentration.

In glutathione, the linkage of the glutamate to cysteine differs from proteins in that the "tail" (side chain γ-CO_2) of the molecule, rather than the "head" (α-CO_2 group), is linked to the cysteine:



This inverted linkage distinguishes glutathione from protein and allows glutathione synthesis and degradation to be controlled separately from protein. The linkage of glutamate to cysteine also decreases the reactivity of the sulfur in cysteine. These two properties allow glutathione to be regulated independently and to be used as a short-term storage form of cysteine. This assures a continuous availability of cysteine for protein synthesis.

Glutathione function as short-term storage form of cysteine: Unlike fat and sugar, which are stored respectively as adipose tissue and glycogen, protein cannot be stored by the human body. For many of the amino acids, the amount of free amino acids in the body approximates the amount required daily for protein synthesis. Because cysteine is reactive and maintained at low concentrations, the amount of free cysteine is limited. The total free cysteine is less than 10 mg/kg body weight, compared to a recommended daily allowance

(RDA) for sulfur amino acids of 21 mg/kg per day. However, the stabilized linkage of cysteine in glutathione provides a storage form for cysteine. The total glutathione is about 150-300 mg/kg body weight, containing 50-100 mg/kg cysteine. The glutathione content of the liver is higher than most other tissues, and liver glutathione is most readily available for release into blood. Glutathione released into blood is converted back to cysteine and its other constituents by the kidneys. The amount of glutathione in the liver is sufficient to maintain about 24-h supply of cysteine. Glutathione in muscle and other tissues is sufficient to provide at least another 24-h supply. However, the body does not allow the pools of glutathione and cysteine to be exhausted during periods of insufficiency. Instead, the body relies upon the large total thiol content of thiols in the body, which is much greater than the glutathione and cysteine pools:

TABLE 1: Comparison of body composition, dietary sulfur amino acid requirements, dietary sulfur amino acid intake, acute toxic dose of acetaminophen, and cumulative dose of thimerosal in terms of total body content of thiol, cysteine equivalents, and glutathione equivalents[a]

Component	Content (mmol/kg body weight)	Cysteine equivalents (mg/kg body weight)	Glutathione equivalents (mg/kg body weight
Total body thiol	20	2400	7200
Total body glutathione	0.8	100	300
RDA for sulfur amino acids (adults)	0.17	21	63
RDA for sulfur amino acids 0-6 mo	0.5	59	180
Sulfur amino acid intake, 2-6 mo, 1%ile to 99%ile Ref wt: 6 kg	0.25 to 2.5	33 to 320	100 to 960
APAP toxic dose, children (150 mg/kg)	0.08	8	24
Thimerosal approx. cumulative dose (200 μg/kg)	0.001	0.1	0.3

[a]Data are approximate values. Total body thiol are based upon older cadaver data which are reasonable based upon tissue measurements of approximately 20 mM total thiols. Cysteine equivalents are calculated based upon the molecular weight of cysteine. Glutathione equivalents are calculated based upon the molecular mass of glutathione being approximately 3 times that of cysteine. Data for the RDA for sulfur amino acids are from the Dietary Reference Intakes (National Academies Press 2005). Sulfur amino acid intake levels are from Third National Health and Nutrition Examination Survey (NHANES III). Acetaminophen toxic dose is the toxic dose for children from Mayo Foundation for Medical Education and Research (www.mayoclinic.com/health/acetaminophen). Approximate cumulative dose for thimerosal is from Ball et al, 2001.

The function of glutathione as a short-term storage form for cysteine has been well-established in rodent studies where liver glutathione concentration undergoes a diurnal

variation linked to dietary intake of sulfur amino acids. Following intake of sulfur amino acids, liver glutathione increases approximately twofold. In post-absorptive periods, the glutathione is released into blood plasma and degraded to provide a continuous supply of cysteine. Studies of human plasma show that a diurnal variation in glutathione occurs with a time course in which the maximal glutathione concentration in plasma occurs at 6-7 h after the maximal cysteine value, consistent with glutathione function as a short-term storage for cysteine.

Glutathione functions in detoxification. Glutathione is an important component of biological systems to protect against toxicity. As indicated above, oxidants, electrophiles, and metals represent three different categories of chemicals that can cause toxicity by damaging sulfur-containing systems. Toxicity caused by each of these categories of chemicals has been studied extensively, but detailed mechanisms often remain incomplete due to experimental limitations and complexity of the biological systems.

Oxidative stress. Oxidative stress is an imbalance of oxidants and antioxidants that results in a disruption of redox signaling and control, or macromolecular damage. Examples of chemical exposures with oxidative stress that affect human health include carbon tetrachloride damage to the liver, paraquat damage to the lung, and doxorubicin damage to the heart. In animal models, each of these chemicals results in decreased tissue glutathione and oxidative damage to macromolecules.

The concepts of oxidative stress as a component of toxicity and disease are currently undergoing considerable revision due to the observations that large-scale double-blind interventional trials with relatively high doses of effective free radical scavenging antioxidants (vitamins C and E) have shown little health benefit. However, relevant to the question of oxidative stress as a mechanism of disease, the data support the interpretation that oxidative stress is a more complex process than implied by a simple imbalance of oxidants and available antioxidants. For example, many of the oxidants that are important in aging and age-related disease also function in useful biologic processes, such as cell communication, defense against microorganisms and tissue turnover, growth and repair. Thus, use of biochemical markers of oxidative stress to infer mechanisms of injury is limited because oxidative biomarkers can reflect a causative oxidative mechanism of injury, activation of the immune system due to infection, allergy or adverse food reaction, or normal signaling during tissue development and growth. Importantly, oxidative species are generated during active cell death in a process known as apoptosis, and cell death by any cause (including normal growth and development) appears to cause increased generation of oxidative markers.

Acceptable experimental tests of toxicity in humans are limited; thus, most evidence for oxidative stress as a mechanism in human disease is circumstantial. This includes observational data in humans, experimental studies in animals, cell culture studies, and mechanistic chemical studies. Despite abundant circumstantial data, large-scale, double-blind interventional trials with antioxidants have generally failed to demonstrate significant health benefits, even though individuals with deficiency in the antioxidant vitamin C clearly show benefit from supplementation.

A critical issue for oxidative stress is to define the meaning of toxic environmental exposures. Some agents exhibit toxicity in which the degree of toxicity is proportional to the dose, while many others show a threshold effect in which no toxicity is observed below a certain exposure. Agents which cause oxidative stress typically have a different characteristic. Low dose exposures of agents which cause oxidative stress activate cell defense mechanisms against oxidative stress. Thus, instead of causing toxicity, these agents actually provide protection against toxicity. This principle has been extensively developed in terms of chemoprevention of cancer, where exposure to phytochemicals from plant-derived foods activates mechanisms which protect against cancer-causing chemicals. This mechanism is specifically relevant to sulfur metabolism because the x_C^- transport system for the cysteine precursor, cystine, and the GCL enzyme system for glutathione synthesis respond to low levels of oxidative stress. Activation of these systems improves protection. The important point relevant to thimerosal is that changes in sulfur metabolism, even if they occur, can reflect activation of protective mechanisms and therefore cannot be simply equated with toxicity. Indeed, changes in sulfur metabolism due to activation of protective mechanisms are likely to be far more common than changes reflective of toxicity, especially given the low exposure doses of thimerosal from routine vaccination.

Metal ion toxicity. Many metals including mercury have a natural affinity for the sulfur in glutathione, and glutathione is known to bind metal ions and to function as carriers to transport glutathione within and between cells. Unless there is a high concentration of metal ion relative to the total thiol pool, there is no way to infer benefit or harm from the interaction of the metal with glutathione. As described above, the body has an abundance of glutathione and other thiol compounds, and the alteration of sulfur metabolism may be a protective mechanism.

Comments on Dr. Deth's Report

The summary provided by Dr. Deth outlines arguments for the general hypothesis that thimerosal present as a preservative in certain vaccines represents an important risk factor for autism, particularly in individuals possessing certain risk-inducing polymorphisms. Key issues identified by Dr. Deth are the distribution and elimination of thimerosal, its metabolic and neurologic actions, and parallels of these findings with metabolic abnormalities found in autistic children. His conclusions are based upon a perceived "congruence between attributes of thimerosal and the features of autism, particularly in individuals possessing certain risk-inducing polymorphisms".

Dr. Deth's summary includes several statements which are generally correct but misleading in the sense that critical issues are not addressed rigorously. For toxicity, dose makes the poison. Consequently, the distribution and elimination studies represent a critical issue concerning the plausibility of the central hypothesis, and rigorous evaluation of this data do not support the conclusion reached by Dr. Deth. Related to this, there is scientific consensus that most mercury-containing chemicals will kill cells in culture at sufficient doses. Accordingly, a key issue for extrapolation of information from such in vitro studies is that

dosing must be adjusted to yield the same final cellular dose, and not the same initial concentration in the extracellular fluid. Cells accumulate metals from the external fluid so that the same extracellular concentration can yield 1000-fold higher cellular load in vitro than in vivo. Thus, in vitro toxicity data are difficult to extrapolate to in vivo toxicity and are not reliable as a basis to determine causality in vivo.

Thimerosal: General Attributes

I believe that the general conclusion stated on Page 2, final sentence, is invalid and misleading: "There is no a priore reason to assume that ethylmercury does not share a similar level of toxic risk as methylmercury, since they are close chemical analogs sharing many physical and chemical properties." This assumption may be reasonable to provide a first approximation of expectations for unknown and unstudied systems, but it is often wrong and is certainly inappropriate when considering 1- and 2-carbon compounds. In biologic systems, one-carbon metabolism and two carbon metabolism are highly specific and use different metabolic pathways. Methylation is a common metabolic control reaction; ethylation is not. Formylation (1-carbon) of methionine is used for translation initiation; acetylation (2-carbon) is not used for this purpose but has other specific uses in biologic systems. Formaldehyde (1-carbon) is metabolized by reaction with glutathione followed by dehydrogenation; acetaldehyde (2-carbon) is metabolized by metabolic pathways which do not require glutathione. Methanol (1-carbon) toxicity is very different from ethanol toxicity (2-carbon), both in terms of dose and effects. Methionine is a required amino acid containing a thiomethyl (1-carbon) group, while ethionine is the corresponding amino acid with a thioethyl (2-carbon) group. Methionine is essential for life while ethionine is toxic to the liver and a carcinogenic chemical. Thus, although there is some merit to the generalization (in a very broad sense) that chemicals sharing physical and chemical properties have similar level of toxic risk, there is little validity for this generalization when used for comparing 1-carbon and 2-carbon metabolites.

The Burbacher et al. 2005 paper comparing blood and brain mercury levels in infant monkeys exposed to methylmercury or vaccines containing thimerosal provides important information concerning brain mercury pharmacokinetics in a primate species. The study shows that mercury derived from the vaccine reaches the brain. However, the study provides no information on toxicity or autism. The authors conclude that "MeHg is not a suitable reference for risk assessment from exposure to thimerosal-derived mercury. Knowledge of the toxicokinetics and developmental toxicity of thimerosal is needed…" Thus, the conclusion of Dr. Deth's discussion of this study "Accordingly, at equal doses, thimerosal carries a higher risk of producing neurological impairments than methylmercury", is a non-sequitur and inappropriate. The study did not address ethylmercury toxicity, and specifically concluded that comparison to methylmercury was inappropriate.

Dr. Deth's final paragraph on the general attributes of thimerosal (p.3) contains similarly disconnected statements. In vitro cell studies are useful for elucidation of possible mechanisms of toxicity because of the ability to carefully control experimental conditions. Thus, studies which show that a certain concentration of a chemical induces apoptosis provide useful information on the pathway of cell death under these conditions. However,

cell studies of toxicity are less useful and, indeed, can be highly misleading concerning dose-response characteristics. A specific limitation for cell studies occurs when cells accumulate toxic chemicals. Chemicals that bind to thiols accumulate in cells. In vitro, the volume of media outside the cells is often 1000 times more than the volume of the cells. In contrast, the extracellular volume in vivo is less than the total cell volume. Thus, for chemicals that bind to thiols, the delivery to cells in vitro is 1000-fold greater than that which would occur with the same extracellular concentration in vivo. The in vitro studies can be further misleading for chemicals which are metabolized or cleared by liver or kidney, or for cells which are privileged in their location behind protective barriers, such as the blood-brain barrier. Thus, experiments need to be designed to evaluate the cellular load of the toxic species to determine whether this is comparable to the cellular load which occurs in vivo.

The last sentences appear to be a random collection of thoughts which may or may not have any relevance to the question of toxicity. Apoptosis is a common mechanism of cell death, so this provides no useful information concerning whether thimerosal causes autism. It is not clear that aberrant apoptosis is a feature of autism. Alterations in urinary porphyrins can occur due to a number of causes, and this issue is not developed adequately to evaluate the scientific validity of the connection intended by Dr. Deth.

Thimerosal and Sulfur Metabolism

Dr. Deth makes two points in the opening paragraph of this section of his report that earlier reviews of thimerosal safety did not address whether thimerosal affected sulfur metabolism and did not address whether sulfur metabolism is altered in autistic children exposed to thimerosal. The specific question raised by the first point is whether thimerosal has significant adverse effects on sulfur metabolism at doses administered. The second point raises the hypothesis that thimerosal causes autism by perturbing sulfur metabolism. I feel that the logic of these questions is not fully developed, but rather needs to be expanded to three questions: Does thimerosal have significant effects on sulfur metabolism at the doses administered? At doses where effects on sulfur metabolism occur, are these adverse effects? Are adverse effects on sulfur metabolism a cause of autism?

With regard to the first question, the dose of thimerosal received from vaccination may be high enough to cause acute changes in local and blood levels of sulfur metabolites, although this question has never been studied. Activation of immune responses can also result in local and blood changes in sulfur metabolism. Changes attributable to thimerosal specifically or vaccination generally are likely to be transient and relatively small, because the dose of thimerosal is relatively small even for an infant (0.1 mg/kg cysteine) when compared to the normal daily intake of sulfur amino acids in the lowest 1 percentile of sulfur amino acid intake (33 mg/kg cysteine equivalents; See Table 1).

With regard to the second question of whether or not the possible effects of thimerosal on sulfur metabolism may be adverse, it is important to remember that, as I previously described, low doses of toxic agents often enhance protection against toxicity by changing sulfur metabolism to favor detoxification mechanisms. Consequently, an acute, localized

change in sulfur metabolism that may occur following thimerosal exposure cannot be said to be an adverse effect and may instead be a beneficial response.

With regard to the third point, specific adverse effects which are mechanistically linked to disease are needed to support the conclusion that thimerosal is a risk factor for autism. This has not been shown. Strong evidence to establish such a link would be especially needed for effects on sulfur metabolism, because genetic, dietary, and environmental variations have effects on sulfur metabolism

Dr. Deth considers 4 critical roles of sulfur metabolism, including 1) maintenance of cellular reduction/oxidation (redox) status, 2) support of methylation (1-carbon transfer) reactions, 3) detoxification and elimination of heavy metals and xenobiotics, and 4) formation of sulfate. In this consideration, Dr. Deth overlooked the most fundamental aspect of sulfur metabolism, namely, that sulfur is an essential component for protein synthesis, and the quantitative requirement for sulfur amino acids for protein synthesis overrides all other aspects of sulfur metabolism. Secondly, Dr. Deth does not clearly elucidate the pathways relative to the nutritional and metabolic precursors. For instance, the methylation reactions are linked to only one dietary source of sulfur, namely methionine, while the redox and detoxification processes are linked to at least 2 dietary sources of sulfur (methionine and cysteine), and sulfation is linked to at least 3 dietary sources of sulfur (methionine, cysteine and sulfate). The metabolic requirements for methionine in methylation reactions are independent of the cysteine requirement for glutathione, which is needed for detoxification and redox reactions. The conversion of methionine to cysteine is unidirectional, and dietary intake of cysteine has little sparing effect on the requirement for methionine. Thus, the admixture of the methionine and cysteine metabolism only confuses rather than clarifies consideration of sulfur metabolism. There is little evidence that perturbations in the downstream cysteine-related metabolism have direct effects on upstream methylation reactions which depend upon methionine. Thus, the argument that something which reacts downstream (with glutathione) has a specific effect on upstream components (methionine) is largely specious.

Methylation, detoxification, and redox processes are commonly altered in association with pathology. Such changes have been clearly demonstrated to occur as a consequence of changes in food selection and food intake, tissue injury and cell death, infection, oxygen deficiency, and other causes. But the admixture of functions (1, 3 and 4) which depend upon cysteine and its product glutathione, with functions (2) which depend upon methionine, only serves to confuse rather than clarify the critical metabolic questions.

The mechanistic arguments for thimerosal perturbations of sulfur metabolism made by Dr. Deth are not sound. Specifically, if defects in sulfur metabolism were causally related to autism, the observational evidence for a role of altered sulfur metabolism in autism indicates an upstream effect in methylation reactions rather than downstream in glutathione-dependent reactions. There are many factors known to regulation methylation pathways, but there is little evidence that downstream events in the glutathione-dependent pathways have a major role in this. Thus, my interpretation is that at relevant doses, thimerosal does not disrupt methylation processes by altering glutathione metabolism.

The possibility that disruption of sulfate metabolism could occur due to thimerosal seems far-fetched without supportive scientific data. Sulfate is an important ion in cells and is present at millimolar concentrations. The amount needed for DHEA sulfation is orders of magnitude lower. Sulfates are also present in the diet. Thus, it does not seem possible that thimerosal could deplete sulfate by the mechanism suggested.

An additional relevant point is that if alteration in glutathione metabolism were sufficient to cause autism, then autism would be expected to be caused by hundreds of dietary, environmental, inflammatory, and infectious agents which affect glutathione metabolism. For example, fasting, starvation, low protein diets, low sulfur amino acid diets or chronic alcohol consumption can result in decreased glutathione. Environmental exposures such as cigarette smoke, ozone and certain aldehydes can decrease glutathione. Viral infections, such as influenza and HIV-1, decrease glutathione, and activation of an inflammatory process is associated with decreased glutathione. Similarly, methylation is commonly altered by a range of dietary and genetic factors. Thus, my opinion is that general similarities in effects, even if they occur, are not sufficient criteria for considerations of a potential causal relationship linking thimerosal to perturbed glutathione to perturbed methylation to autism.

Effects on Cellular Redox Status

The concept of cellular redox status as used by Dr. Deth is oversimplified. There is no single redox status; the multiple thiol/disulfide systems in cells and different tissues are not equilibrated, and these values change as a function of time of day, diet, age, and other factors. Glutathione values are usually maintained within a certain range, but can vary considerably. These variations can occur without corresponding changes in glutathione redox status, because both the oxidized and reduced forms of glutathione change. Cell and animal studies show that considerable decreases in glutathione can occur without killing cells. For example, buthionine sulfoximine is a chemical that inhibits glutathione synthesis and is used experimentally to decrease glutathione. Studies show buthionine sulfoximine treatment of mice and rats depletes many tissues of glutathione by 80-90% and increases sensitivity to various types of toxicity, but that reduction in glutathione alone does not cause toxicity. Toxicity with buthionine sulfoximine alone occurs only with high doses. In very young mice, high dose buthionine sulfoximine causes cataract formation. This experimental model complements what we know from acetaminophen toxicity, namely that there is considerable tolerance to perturbations in glutathione metabolism so that a perturbation of glutathione is unlikely to be the mechanism of autism.

As stated above, my interpretation is that thimerosal at the doses used in vaccines is not likely to induce the magnitude of decrease in glutathione needed to induce toxicity. Dr. Deth concludes that thimerosal causes toxicity in vitro in the low nanomolar range. I have therefore reviewed the in vitro evidence concerning the doses which cause toxicity:

> In a study by James et al 2005, 10 μmol/L of thimerosal given to neuroblastoma and glioblastoma cells killed approximately half of the cells. This dose is equivalent to 2 mg/l, or 2 μg/ml. Assuming cell wet weight is 1 mg/ml, this dose would be 2 μg/mg tissue, or 2 mg/g tissue or 2 g/kg tissue. Exposures to thimerosal from vaccination

may reach approximately 200 μg/kg tissue (Ball et al, 2001); thus, the concentration which caused toxicity in the neuroblastoma and glioblastoma cells was >1000-fold higher than the maximal dosing in vivo from routine childhood vaccination.

Other in vitro studies involving thimerosal include Park et al (2007), who found that 2.9 μM killed 50% of mouse inner medullary collecting duct cells, while 9.5 μM killed 50% of human embryonic kidney cells (HEK293). The concentration of thimerosal which induced apoptosis in 50% of SCM1 gastric cancer cells was between 5 and 10 μM (Liu et al 2007). The cJun N-terminal kinase pathway, which has been associated with activation of cell death by apoptosis, was activated in a neuroblastoma model at 2.5 μM thimerosal, but not 1 μM thimerosal (Herdman et al, 2006). A similar range of concentration of thimerosal (2.5 and 5 μM) was found to cause cell death in human neuroblastoma cell line SK-N-SH (Humphrey et al 2005). Parran et al (2005) studied effects of thimerosal on nerve growth signal transduction and cell death in neuroblastoma SH-SY5Y cells. They showed that the thimerosal concentration required for 50% cell death was 596 nM in the presence of nerve growth factor and 39 nM in the absence of nerve growth factor. They concluded that nerve growth factor provides protection against thimerosal-induced cytotoxicity.

Thus, there is abundant evidence that thimerosal kills cells in culture when administered in the low micromolar range, not low nanomolar range as indicated by Dr. Deth. It is not clear where Dr. Deth's purported threshold value for thimerosal reduction of glutathione (0.1 nM) was obtained, because this range is >5 orders of magnitude (>100,000-fold) different from the value of 15 μM thimerosal which caused about 40% decrease in glutathione in glioblastoma cells (James et al, 2005). Of course it is possible to manipulate cells in culture to increase sensitivity, but it is unlikely to be able to detect a change at that low concentration because this would require the ability to detect 0.1 nM change in glutathione concentration, and there is no current technology with sufficient sensitivity to measure this extent of loss in biologic systems.

Other issues are relevant to the question of whether sufficient oxidative stress is induced by thimerosal at the doses used to cause toxicity in vivo. Glutathione is present at a relatively high concentration in cells, but the reservoir for metabolic reducing equivalents to protect against oxidative stress is not provided by glutathione, per se. Glutathione is part of a reductase system which obtains reducing equivalents from NADPH; substrates which provide reducing equivalents for NADPH are the reservoir for reducing equivalents. In most cell types, glucose is a major substrate for this pathway through the pentose phosphate pathway. Direct measurements of NADPH supply show that this rate greatly exceeds the rate of glutathione biosynthesis. This means that the capacity to handle oxidative stress is even greater than that indicated by the high capacity to synthesize glutathione. Studies of liver cells show that rates of peroxide metabolism up to 20% of the total cellular respiration (O_2 consumption) rate can be accommodated before decreased glutathione occurs (Jones et al 1981; Jones 1982). By comparison, two-fold changes in glutathione content occur during a 12-h period due to the eating and fasting cycle in mice and rats. Thus, the reductive capacity available from the glutathione system to protect against oxidative stress is large, and the function of this system is not limited by the size of the glutathione pool.

Oxidative changes characteristic of oxidative stress are a common consequence of disease processes. Oxidation occurs as a consequence of poor food intake, from illness and from tissue injury, inflammation, and cell death. Oxidative stress is also a part of the body's mechanisms that respond to infection and in wound healing and repair. Thus, one must use caution in the interpretation that oxidative stress is a cause of disease, as it may actually be the body's response to the disease. Thus, potential efficacy of agents to decrease oxidative stress and improve outcome in autism is not sufficient to conclude that thimerosal alters glutathione, altered glutathione alters oxidative stress, and oxidative stress causes autism.

Furthermore, interpretation of oxidative stress markers which have been associated with autism is complicated by drug and supplement use. For instance, in the comparison of autistic and control children in the study of Ming et al (2005), autistic children were receiving a range of different medicines, including Lamotrigine, Pimozide, Guanfacine, Topiramate, Risperidone, Valproate, Sertraline, Clonidine, Methylphenidate and Gabapentine. Four of the 33 autistic children were receiving supplements, including fish oil melatonin and probiotics. Because drug therapy can result in altered glutathione and oxidative stress markers, the results do not allow unambiguous interpretation of the association of oxidative stress markers with autism. Treatment addresses symptoms of disease, not cause of disease. Thus, even if therapies for sulfur metabolism improve autistic symptoms, there is no logical conclusion one can draw from the efficacy of treatment of disease concerning its underlying cause.

Dr. Deth links together many isolated pieces of information concerning different pathways, model systems, and mechanisms without a clear logic in presentation or rigorous evaluation. Hence, I have found myself doubling back on issues trying to cover them all. In the end, I must say that I feel that the burden of proof that thimerosal in vaccines causes autism must be borne by those who make this claim. From my perspective, the arguments are weak at best. The primate model of Burbacher et al 2005 showed clear evidence for delivery of mercury to the brain following injection. Beyond this, the argument that the dosing is sufficient to cause toxicity of any sort by perturbing the glutathione system is not justified by what is known and generally accepted about the glutathione system. The argument that perturbations in methylation pathways are associated with autism creates a false linkage of glutathione to autism because glutathione is downstream of methionine in the sulfur metabolism pathway. Although there is some possibility for the downstream glutathione to have effects on methylation, methylation is responsive to many different dietary and genetic factors, and there is not reliable evidence that glutathione has a primary role in controlling methylation. Thus, there are significant gaps in the argument that mercury from thimerosal causes a sufficient perturbation in glutathione to result in specific defects in methylation pathways to account for the neurodevelopmental abnormalities of autism.

Genetic Vulnerability to Thimerosal

The advances in understanding genetic risk factors associated with autism are noteworthy, but the potential links between genetic variations and the hypothesized connection of thimerosal and autism appear to be completely conjecture. Oftentimes, polymorphisms have

no effect on measured activities and require experimental verification. Data are not provided to show that these polymorphisms change critical parameters. Critical data are missing to show that individuals with these genetic variants who received thimerosal-containing vaccines had higher incidence of autism than those who did not. Thus, this conjecture is lacking scientifically valid supporting data and is largely a waste of time. If there are data to support the point, those should be presented. Otherwise, this simply demonstrates that progress is being made in providing genetic bases for autism—a fact that discredits the argument that the cause is thimerosal in vaccines.

Sincerely,

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