Respondent's Exhibit Q

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Re: Thimerosal and Autism

I have been asked by the Department of Justice to evaluate documents submitted by Dr. Richard Deth regarding a potential scientific link between thimerosal (ethylmercury thiosalicylate) and autism. My qualifications and background are discussed below.

I received a B.S. (1984) in Biology (minors - Chemistry and Philosophy) and a M.S. (1986) in Pharmacology (minor – Biochemistry) from the University of Minnesota-Duluth. My Ph.D. (1992) in Molecular and Environmental Toxicology (minor – Cancer Biology) was done at the University of Wisconsin-Madison. Postdoctoral training (1992-1995) in Molecular Neuropharmacology at the University of Washington-Seattle was followed by an Assistant Professor appointment (1995) in the Department of Pharmacology, Toxicology and Therapeutics at the University of Kansas Medical Center. After four years, I moved back to the University of Wisconsin as an Assistant Professor in Pharmaceutical Sciences (1999). Promotion to Associate Professor with tenure was awarded in 2003 and to Full Professor in 2007. I have received continuous grant support from NIH as well as from numerous foundations and the State of Wisconsin. The direct cost of funds acquired over the past four-year period exceeds $3,100,000. In that same period, 28 peer-reviewed manuscripts were published with a career total of 54 publications.

The focus of my research program is neuroprotection, neuropharmacology and neurotoxicology. Specifically, we are trying to modulate oxidative stress in chronic neurodegenerative diseases to slow and/or stop disease progression. Models of Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis (Lou Gerhig's disease), Huntington's disease, and multiple sclerosis are currently being used to investigate/identify novel neuroprotective pathways that could lead to the development of new therapeutics. Some of our recent work has garnered national attention. In studies using transgenic models of AD, we discovered a possible new therapeutic approach for treatment of this devastating disease. Furthermore, the Nrf2-ARE pathway was identified as being critical to blocking oxidative stress-induced neuronal cell death both *in vitro* and *in vivo*.

The public exposure of our work over the past two years has allowed me to become active in many local support groups for neurodegenerative diseases. I have spoken at ALS support group meetings and interacted with patients and their families at numerous social events. I also spoke to the Wisconsin chapter of the Huntington's Disease Society of America and was presented with the Community Humanitarian Award by the

Great Lakes Region of the Huntington's Disease Society of America (2006). I am the Director of the Pharmacology and Toxicology B.S. Program (2002-present) and served as Associate Director (2003-2005), as well as Director of the Molecular and Environmental Toxicology Center (2005-2006). I have also had a wide variety of committee appointments at the level of the Division, School and University. Outside of the University, I was a member of the NIH Neurotoxicology and Alcohol (NAL) study section for five years (2002-2007).

With this background and experience, and after an extensive review of the literature cited and newly published data regarding thimerosal, I conclude that there is not sufficient evidence to support an association between thimerosal administration and autism. In fact, there is highly significant and scientifically sound data to make the opposite conclusion – **there is NO link between thimerosal and autism.** The subsequent pages give a detailed analysis of the rationale behind this conclusion.

Prior to discussing Dr. Deth's statements, two highly relevant new publications must be pointed out and their findings discussed:

1. Dr. Robert Berman at the University of California-Davis published a new paper in January 2008 looking at thimerosal in the SJL/J mouse strain (Berman et al., 2008). Berman chose this mouse strain and the experimental design because it mimicked the vaccine dose schedule for humans in early postnatal mice as well as the experiments done by Hornig et al. [2004 (Deth ref. 26)]. Mice were dosed on postnatal day 7, 9, 11 and 15 with a cumulative dose of thimerosal equaling 39.8 µg/kg (the dose on a per kg basis that a child would receive through vaccinations) and 398 µg/kg (a dose 10 times higher to clearly show if toxicity occurred). Numerous behavioral, morphological, and histological outcomes were measured. In addition, Hg levels in blood, brain and kidney were also determined. The choice of the mouse line, days of exposure and 39.8 µg/kg dose were based on earlier studies that *suggested* thimerosal had significant toxic effects with regard to behavioral, morphological, and histological outcomes in the SJL/J mouse and not in the two other strains tested [Hornig et al., 2004 (Deth ref. 26)]. I have thoroughly examined the outcomes in both papers and support the conclusion of Dr. Berman. In general, the quality of the data in the Berman paper far exceeds that in the Hornig paper. In my opinion, the hippocampal sections and staining in the Hornig paper are the poorest quality I have ever seen published and they could easily lead the authors to make invalid conclusions. This is in stark contrast to the data presented in the Berman paper. The images in this paper are of the highest quality and the conclusions are quite clear. The implied brain damage induced by thimerosal in the Hornig paper at the cumulative dose of 39.8 µg/kg was not validated in the Berman paper. Even more importantly, no brain damage was seen at the 398 µg/kg dose in the Berman paper. The fact that a ten times higher dose had no significant effects is a striking observation that completely invalidates the findings by Hornig. The other major difference is that Hg levels were not determined in the Hornig paper. The lack of these data bring into question what the actual levels of Hg were in the Hornig study and further invalidate the study's conclusions.

2. Schechter and Grether (2008) published an article entitled, “Continuing Increases in Autism Reported to California’s Developmental Services System: Mercury in Retrograde.” Autism rates in California have not decreased despite the virtual complete lack of thimerosal in vaccines beginning in early 2001. The overall conclusion of this large study of children between 3 and 12 years of age was that exposure to thimerosal during childhood is not a primary cause of autism.

In my opinion, these two findings are truly the last “nails in the coffin” invalidating the proposed link between thimerosal and autism. However, some continue to purport the theory based on more and more inconsistent scientific hypotheses that are yet lacking the support of significant scientific data. I will attempt to bring clarity to these issues and address the contents of Dr. Deth’s report.

In the beginning of his report, Dr. Deth implies that there is no *a priore* reason to assume that ethylmercury does not share a similar level of toxic risk as methylmercury (MeHg), since they are close chemical analogs sharing many physical and chemical properties. Although these two molecules differ by only one methyl group, Dr. Deth’s assumption about their potentially similar toxic risks is not valid, as evidenced by another pair of molecules that differ by only one methyl group with dramatically different effects on the human body – methanol and ethanol. While ethanol is a type of alcohol that can be found in red wine, methanol is lethal. Therefore, the extensive amount of work done using MeHg toxicity *cannot* be used as the background information supporting the toxicity of ethylmercury.

Dr. Deth then proceeds to discuss thimerosal toxicity in relation to human cortical neurons and neuronal cells in culture. The greatest Hg levels reached in the brain of primates [Burbacher et al., 2005 (Deth ref. 2)] and mice (Berman et al., 2008) dosed with relevant levels of thimerosal were 30 ppb (Burbacher et al. 2005; Fig. 6) and 9 ppb (Berman et al. 2008; Table 2), respectively. These numbers would equate to a concentration of 30 or 9 nM in solution. It should be noted that the dosing in the primates was a total of 80 µg/kg whereas the mouse experiments were half that at 39.8 µg/kg. The papers referenced regarding neurotoxicity in culture need 2.5-10 µM [Herdman et al., 2006 (Deth ref. 3)], 1-250 µM [Baskin et al., 2003 (Deth ref. 4)] and 0.1-1 µM [Parran et al., 2005 (Deth ref. 5)] to show significant cell death, which are levels much greater than occur *in vivo*. The cells used in these assays were *not* neurons or neuronal cells, contrary to Dr. Deth’s statement. They are constantly dividing cell lines that clearly do not represent either primary neurons in culture or neurons in the brain.

Even if I were to agree with Dr. Deth that oxidative stress and redox status appear to be altered in autistic patients based on the two papers by S. Jill James [James et al. 2004 (Deth ref. 9); James et al. 2006 (Deth ref. 10)] and the other papers referenced (Deth ref. 12-19), none of this evidence links or even allows one to speculate that oxidative stress, redox status and autism are associated with thimerosal exposure. Both oxidative stress markers and a neuroinflammatory response are common to many neurological disease states and are in no way isolated to autism. In the majority of cases, these are seen as reactions to a pathologic process and progression of the disease. It is interesting that in **all other** diseases of the brain with oxidative stress and

neuroinflammation, the disease progresses, leading to eventual neuronal cell loss and death. This does not appear to be the case in autistic patients.

Dr. Deth continues by trying to link thimerosal to oxidative stress, and references Makani et al [2002 (Deth ref. 21)]. This work uses a Jurket T cell line and µM dose to show effects, which are both irrelevant to any *in vivo* situation. Cell death after 24 hrs is one of the endpoints. The GSH levels were measured at 18 hrs and were found to be reduced. At this time, however, the majority of cells are dead or dying, so of course GSH levels will be reduced. GSH levels were not determined at earlier time-points making these data difficult to interpret. James et al. [2005 (Deth ref. 22)] is also referenced and, again, cell lines are used – a neuroblastoma and glioblastoma cell line – and doses of thimerosal are µM. The dose used to generate the GSH data was 15 µM – an extremely high dose. In 3 hrs that dose kills approximately 75% of the cells. The GSH levels were determined 1 hr after dosing. These cells are figuratively being hit with a "sledge hammer" and the data are meaningless. Overall, the cells, as well as the doses used in this paper, have no relevance to the *in vivo* situation. The authors themselves state that the acute high doses of thimerosal used were "not intended to mimic exposures of developing brain cells *in vivo* to thimerosal vaccines" [James et al. (Deth ref. 22)].

One reference to Dr. Deth's own work (Deth ref. 24) is listed as "manuscript in preparation." To date, these data referenced in Dr. Deth's report have not been published in a peer-reviewed journal, nor were they, or the unpublished manuscript, submitted with his report in this case for my review. Therefore, I am unable to comment on whether the data Dr. Deth references is relevant to the case or not. The doses of thimerosal that Dr. Deth indicates as having negative biological outcomes are 100 to 1000 times lower than any other published data. Some of these other publications testing thimerosal actually use the same cell line as Dr. Deth, and need to use far higher doses to see negative biological outcomes. This huge differential cannot be accounted for through biological error (such as the cells being different), because they are the same cells. One could see a 5 or maybe 10 fold difference due to biological error when treating with thimerosal that could be due to growing the cells in different medium (a solution used to make the cells grow and survive), or cell density [if you look at the dish the cells are growing on and there is space (low density) or no space (high density) between neighboring cells]. A 100 to 1000 fold change in sensitivity is not possible. Therefore, the most likely explanation for this phenomenon is technical error, meaning error associated with the experimenter (math mistakes, dilution mistakes, volume mistakes, incorrect solution mixtures, etc).

As discussed earlier, the data presented by Hornig et al. [2004 (Deth ref. 26)] has been refuted by Berman et al (2008). Dr. Deth refers to the hypothetically sensitive strain of mice in this paper as, "the strain harboring genetic deficits in redox-related enzymes." This is absolutely not the case. This is the same SJL/J mouse strain referred to earlier that is supposed to be very sensitive to thimerosal. This mouse strain has some different genes (not deficits) involved in immune response that make them have a stronger immune response to toxins. These genes are called major histocapability complex (MHC) genes and this is why they are used in a variety of studies. The comment above by Dr. Deth implying that these mice have deficits in redox-regulated

enzymes was not even mentioned in the paper he references to support it [Hornig et al. 2004 (Deth ref. 26)]. He is quite wrong in his portrayal of the rationale behind using this mouse strain.

Dr. Deth suggests the importance of redox regulation of the enzyme methionine synthase in converting homocysteine to methionine. A huge assumption is made in order to justify this discussion – Dr. Deth assumes that the metabolite profiles showing reduced methionine and S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH) ratios [James et al. 2004 (Deth ref. 9); James et al. 2006 (Deth ref. 10)] are due to oxidative stress-mediated inhibition of methionine synthase. It should be noted that neither the activity nor level of methionine synthase was measured in the autistic patients from these studies. Based on this assumption, Dr. Deth references his own work, which is the only published work regarding thimerosal and inhibition of methionine synthase. Dr. Deth states, "my laboratory demonstrated potent inhibition of neuronal methionine synthase by thimerosal (27), with half maximal inhibition occurring at approximately 1 nM, 30-fold below the plasma concentration produced by a thimerosal-containing vaccine" [Waly et al. 2004 (Deth ref. 27)]. There are a number of significant issues with the way these data are generated and interpreted, which are as follows:

1. The lab is not measuring **neuronal** methionine synthase. They are measuring methionine synthase activity in a human neuroblastoma cell line derived from a metastatic peripheral nerve tumor. The cells are not normal and have aberrant or mutated forms of methionine synthase that respond differently to treatment. It is likely that this caused a dramatic impact on how the data was interpreted, due to a number of possible resultant factors. One factor is that the level of expression and/or amount of methionine synthase could be dramatically lower than the levels in the brain cells, which could make a big difference in how they respond to treatment. Or perhaps normal cofactors (other proteins required for stability, enzymatic activity, or resistance to toxins) are no longer present in the cell line but are present in normal brain cells. The number of variables between normal brain cells and cell lines is endless, and that is why data generated in cell lines are *only* relevant to that cell line.

2. The assumption that these data represent the *in vivo* human situation is wholly inappropriate. There are currently no *in vivo*, or even primary neuronal culture experiments, to evaluate Dr. Deth's theories.

3. All scientific observations need independent validation. This paper was published in 2004, yet no other laboratory has published data validating Dr. Deth's experiments.

4. Appropriate dose curves were not run for thimerosal to determine its effect on methionine synthase enzymatic activity (Table 1). Running dose curves is important so you can find the best dose to use in the experiment. In this case specifically, a dose curve for $HgCl_2$ and thimerosal should be run to provide the rationale for picking the single dose used in the experiment. As it stands, no rationale is presented as to why 10 µM $HgCl_2$ was used versus 10 nM thimerosal to inhibit methionine synthase (Table 1). The lack of a sound rationale for the

doses used brings into question, and leads one to speculate on, the validity of the data.

5. No clear indication of time of incubation after adding radiolabel is given for the experiments (Table 1), which raises a number of questions (e.g. Is the reaction reversible or irreversible? What was the rationale for the time used in the experiments?) This is important because the cells can adapt and change in response to treatment. For example, we and others have published that you can treat cells with a chemical, then look at glutathione (GSH) levels in the cell at 4 hours and 24 hours after treatment and get completely opposite results. The chemical we treated the cells with binds to GSH, so when you measure the GSH levels at 4 hours, the levels are reduced to 20-30% of normal. But if you wait 24 hours before measuring GSH, the levels can be *increased* to 150-200% of normal. The cell, having sensed the reduction in GSH levels, responds by rapidly increasing the expression of genes that make the proteins (enzymes) that synthesize GSH. The end result is that the cell has not only returned the levels of GSH to normal, but has actually *increased* the levels to higher than normal. This is why the time is important. If the methionine synthase activity is only inhibited at 4 hours and is completely normal or higher at 24 hours, the interpretation of the data becomes quite different.

6. Data were not statistically analyzed (Table 1).

7. The use of [^{14}C] formate is not directly measuring methionine synthase activity. This makes interpretation of these data difficult. The formate must be converted through multiple steps to 5-methyltetrahydrofolate. Then the 5-methyltetrahydrofolate is used by methionine synthase to make methionine from homocyteine. There is the potential that other steps in the process could be interfered with that have indirect effects on what is trying to be measured.

8. The central focus of the paper is to show that thimerosal inhibits methionine synthase by inhibition of the enzymes PI3-kinase and MAP-kinase. The use of selective chemical inhibitors of enzyme activity can be complicated by non-specific effects of the chemicals that can lead to misinterpretation of the data. There are far better and more direct methods to inhibit the enzymatic activity of PI3-kinase and MAP-kinase in cell lines. One very nice way is to use a technique called silencing RNA (siRNA) to prevent the proteins from being made in the cell. This blocks production of the specific protein you want to target letting you make a direct connection between the PI3-kinase or MAP-kinase and inhibition of methionine synthase. If these protein are significantly reduced or eliminated from the cell and thimerosal no longer inhibits methionine synthase then a link has been established. It is also important to know if you are actually blocking enzymatic activity in these experiments. Dr. Deth presents no data measuring PI3-kinase or MAP-kinase activity in the presence of thimerosal. These data have never been independently validated in other laboratories.

9. The discussion and conclusions discussed in comment 8 have a significant impact on Dr. Deth's proposed method of thimerosal toxicity. According to Dr.

Deth, thimerosal is inhibiting methionine synthase by inducing oxidative stress. In this paper he is arguing that thimerosal is a chemical inhibitor of PI3-kinase and MAP-kinase as is shown in Fig. 7 [Waly et al. 2004 (Deth ref. 27)], leading to inhibition of methionine synthase. No connection is made between the two hypotheses. These kinase enzymes are rapidly activated and inactivated. A sustained activation of either of these enzymes is often associated with cancer formation and many other normal pathways in the brain. In fact, activation of PI3-kinase in neurons is one of the best ways to protect the neuron from oxidative stress-induced cell death. It is highly unlikely that there is sustained activation of either of these enzymes in the brain after thimerosal exposure, so it is unclear to me where Dr. Deth is heading with this hypothesis. The results of this study are perplexing, and do not support Dr. Deth's hypothesis.

10. As previously stated, the manuscript listed as Deth reference 24 and any data it contains are not published; therefore, any reliance on this reference and the data is irrelevant.

Based on his assumption that methionine synthase activity is reduced in autistic patients, Dr. Deth continues to speculate that, "these observations are consistent with thimerosal-induced oxidative stress as a cause of autism-associated neurocognitive deficits." A number of other correlations are proposed with regard to phospholipid methylation, DNA methylation, inactivation of neurotransmitter, regulation of nitric oxide synthesis, inhibition of cysteine dioxygenase activity and reduced sulfate availability. The basis of these correlations has no scientific support and is purely speculative. This kind of association would suggest that anything that causes oxidative stress leads to the development of autism. If this were the case, then we would all be autistic because we have all been exposed to oxidative stress from conception to death. Common examples of oxidative stress include conception itself, metabolic byproducts of what we eat, exercise, dieting (caloric restriction), fever, smoking, exhaust fumes, alcohol, injury, and oxygen deprivation.

Overall, this single published observation in a cell line (Deth ref. 24) is scientifically insufficient to justify the hypothesis put forth by Dr. Deth. The concept that changes in autistic patients with regard to oxidative stress, transsulferation, and transmethylation are the result of vaccines containing thimerosal, based on these data, is inaccurate and lacking any sound scientific support.

Dr. Deth states that, "[w]hile the amount of vaccine-derived thimerosal (e.g. 25 µg/dose) is too small to cause a stoichiometric depletion of plasma GSH, thimerosal interferes with cellular production of GSH, contributing to the approximately 40% lower plasma levels in autistic children [James et al. 2004 (Deth ref. 9); James et al. 2006 (Deth ref.10)]. This statement is an oxymoron. First Dr. Deth says that the amount of thimerosal given during vaccination is not sufficient to reduce GSH levels in plasma. Then he implies that thimerosal is responsible for a 40% reduction in GSH levels in autistic patients. If GSH levels are, in reality, at the significantly reduced levels reflected in Dr. Deth's report, and if reduced GSH levels are reflecting a cause of autism, then thimerosal cannot be a causative factor, as it would have little impact at all on GSH. The reduced level of GSH in autistic patients reflects a pathologic process independent

of the thimerosal exposure. Similar kinds of data have been associated with virtually all neurodegenerative disease, as well as the normal aging brain, and are not selective to autism. These kinds of data reflect the effect, not the cause, of the disease. Another major concern with the James et al. 2004 and 2006 studies (Deth ref. 9 and 10), is the lack of any information on caloric intake and diet. The parameters measured, including GSH levels, can be highly dependent on diet.

Attempting to associate these genetic and/or biochemical differences with susceptibility to thimerosal is extremely premature and invalid. Mutations and single-nucleotide polymorphisms (SNPs) associated with autism cannot be associated with thimerosal exposure as Dr. Deth implies. There is no rational scientific reasoning that can justify such an association, and Dr. Deth's single publication in a human neuroblastoma cell line is the sole evidence he even offers in attempting to establish justification.

As mentioned earlier, I conclude that there is not sufficient evidence to support an association of thimerosal administration and autism. In fact, there is highly significant and scientifically sound data to make the opposite conclusion – **there is NO link between thimerosal and autism.**

Sincerely,

Jeffrey A. Johnson

References

Berman RF, Pessah IN, Mouton PR, Mav D, Harry J. Low-Level Neonatal Thimerosal Exposure: Further Evaluation of Altered Neurotoxic Potential in SJL Mice. Toxicol Sci. 2008 Feb;101(2):294-309.

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