Respondent's Exhibit CC

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February 21, 2008

Expert Report

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Qualifications:

I am an MD physician/scientist in the Division of Clinical Pharmacology at Vanderbilt University. I received my MD from the University of Iowa in 1969 where I was elected to Alpha Omega Alpha Honor Medical Society. After receiving my medical degree, I did an internship at Denver General Hospital from 1969–1970. I then served as a Flight Surgeon in the U.S. Navy from 1970–1973. I did a residency in internal medicine at Washington University in St. Louis from 1973-1975 and completed a post-doctorate fellowship in Clinical Pharmacology at Vanderbilt University from 1975–1977. In 1977, I was appointed Instructor in Pharmacology and Medicine and was promoted to Assistant Professor in 1978, Associate Professor in 1983, and Professor in 1986.

I have been elected to the American Society for Clinical Investigation and the Association of American Physicians. I have served on the International Advisory Committee of numerous international conferences. I have been a member of the Scientific Advisory Boards of Cell Therapeutics, Inc., Galileo Laboratories, Kronos Longevity Research Institute, and Lipoprotein Diagnostics. I was also a member of the NIH Medical Biochemistry Study Section.

Currently, I am an Associate Editor of the journals *Prostaglandins & Other Lipid Mediators* and *Free Radical Biology & Medicine.* I was the recipient of the Burroughs Welcome Scholar Award in Clinical Pharmacology (1983), the Sidney P. Colowick Faculty Research Award (2001), a National Institutes of Health MERIT Award (2001), the Discovery Award from the Society of Free Radical Biology & Medicine (2006), the Earl Sutherland Prize for Achievement in Research from Vanderbilt University (2006), the Recipient of the T. Edwin Rogers Chair in Pharmacology (2006), and a Distinguished Alumni Award from the University of Iowa School of Medicine (2007).

I was in the area of prostaglandin research beginning in 1975, but since we reported our discovery in 1990 that prostaglandin-like compounds (termed

isoprostanes) are formed *in vivo* by a non-enzymatic free radical mechanism, my entire research focus has been in the area of oxidative stress and oxidative injury. The importance of that discovery to the field of free radical research has been recognized by my being appointed an Associate Editor in 2003 of the leading journal in the field, *Free Radical Biology & Medicine*, and being the recipient of the Discovery Award from the Society of Free Radical Biology & Medicine in 2006. I was the Keynote Speaker at the First European Workshop on Isoprostanes in 2004. A detailed summary of my experience, publications, and qualifications can be found in my *curriculum vitae*, which is attached to this report as Exhibit DD.

I have reviewed the document from Richard C. Deth, PhD, entitled: "The Relationship Between Thimerosal and Autism." I will limit my opinions to Dr. Deth's proposed causative relationship between thimerosal contained in vaccines and oxidative stress/damage as it relates to autism.

A. Review of literature relevant to a potential relationship between oxidative stress and autism

In 2006, Chauhan, A and Chauhan, V published a review in *Pathophysiology* entitled "Oxidative Stress in Autism" [1]. The authors describe potential mechanisms of oxidative stress in autism; noteworthy is the use of the word "potential." The potential mechanisms they describe in this paper are general mechanisms that can lead to oxidative stress in many diseases. The authors also give references for alterations in levels of antioxidants, antioxidant enzymes, and antioxidant enzyme activities. These include alterations in the ratio of glutathione (GSH) and reduced glutathione (GSSG) in plasma. However, the difficulties and unreliability of measuring GSH and GSSG are well-recognized [2,3].

Moreover, the data linking alterations in the activity of antioxidant enzymes, *e.g.*, superoxide dismutase and glutathione peroxidase, with autism are contradictory [4,5]. A major problem with such measurements is that they are very indirect – alterations in the GSH:GSSG ratio and antioxidant enzymatic activity determinations are not direct indicators of oxidative damage. In particular, GSH and GSSG levels can be affected independently of each other (one may increase or decrease without the other being altered), and this ratio is constantly changing. Furthermore, GSH levels are affected by hundreds of factors, including diet. One cannot, then, make extrapolations about oxidative damage or some underlying oxidant insult by simply observing an altered GSH:GSSG ratio.

Oxidative damage can only be firmly established by measurement of biomolecules that are products of oxidative damage, *e.g.*, products of lipid peroxidation. Chauhan and Chauhan cite two references that made such measurements as evidence of oxidative damage/injury in autism [6,7]. In the paper by Chauhan et. al. 2004, levels of malondialdehyde (MDA) in plasma were measured by the TBARS assay. MDA is a product of lipid peroxidation; however, it is also enzymatically formed by thromboxane synthase. It is well-recognized that measuring malondialdehyde in plasma is fraught

with artifacts, because platelets are activated during blood sampling that release MDA formed enzymatically as a product of thromboxane synthase. Thus, the MDA measured in plasma cannot be controlled for so as to only measure products of oxidative damage, and thus such a measurement is not a specific indicator of lipid peroxidation [8].

Secondly, measuring MDA by the TBARS assay is also recognized to be very problematic since the TBARS assay is not specific for MDA [9]. Overall, use of the TBARS assay to assess oxidative injury has been shown to be unreliable [10]. In spite of these serious problems associated with using the TBARS assay as a measure of lipid peroxidation in plasma, the mean differences in level of TBARS observed between autistic subjects in siblings in the Chauhan paper was very modest (~ 25%), which has questionable biological and clinical relevancy.

It has been firmly and independently established that the most reliable biomarker of oxidative stress/damage is measurement of F_2-isoprostanes, which are products of lipid peroxidation, by gas chromatography mass spectrometery [10]. Dr. Deth cites Ming et al. 2005 in which the authors measured F_2-isoprostanes and the product of oxidative DNA damage, 8-hydroxy-2-deoxyguanosine (8-OHdG), in autistic subjects, but did not use gas chromatography mass spectrometery. They found a significant increase in the urinary excretion of F_2-isoprostanes in autistic subjects compared to controls, but noted that the levels of F_2-isoprostanes were highly variable. However, they did not observe an increase in urinary excretion of 8-OHdG in autistic subjects. As mentioned, it has been established that measurement of F_2-isoprostanes by gas chromatography mass spectrometry is a very reliable approach to assess oxidative stress/damage. However, Ming and colleagues measured urinary F_2-isoprostanes using an immunoassay kit. This is <u>very important</u>, because measurement of F_2-isoprostanes by immunoassay has been found to be scientifically unreliable [11,12]. The wide variances in the values Ming obtained may well stem from the unreliable testing techniques employed.

Other problems with the studies relied upon by Dr. Deth to establish that autistic children exhibit evidence of oxidative stress is that the number of subjects studied is small and confounding factors that may influence the results were not controlled for, including dietary intake of antioxidant-containing foods and level of physical activity, both of which can affect an individual's level of oxidative stress.

In short, there is little if any evidence to conclude that, in general, children with autism experience chronic oxidative stress. However, even if one were to establish that autistic children do have elevated measurements of biomarkers for oxidative damage, this in no way provides insight into the <u>cause</u> of the oxidative damage. Oxidative stress can be caused by many factors, and is in no way exclusive to a toxic insult. Oxidative stress, for example, if it is occurring, could simply reflect some underlying neurological disease process without necessarily being the causative agent of the disease. Markers of oxidative stress are seen in many neurological conditions. I am very familiar with the scope of research in the field of oxidative stress and it is not accepted in this field that autism is caused by oxidative stress.

B. Critique of the report from Richard C. Deth, PhD, entitled "The Relationship Between Thimerosal and Autism"

I will primarily addresses issues/arguments mentioned under **1. Effects on Cellular Redox Status** on page 4-7. Dr. Deth, like in the Chauhan and Chauhan review mentioned above, cites to references that report altered ratio of GSH:GSSG and increased levels of biomarkers of oxidative stress in autism as evidence of oxidative stress in autism. As I explained above, an altered GSH:GSSG ratio is not a reliable finding from which to extrapolate about oxidative insult.

If Dr. Deth is right, namely that oxidative stress is the causative agent for some children developing autism, then oxidative stress caused by other events should also be responsible for cases of autism. Oxidative stress is not an uncommon phenomenon; it can be caused by childhood illness, viral infections, and exercise, to name a few examples. According to Dr. Deth's hypothesis, every child who suffers from a viral infection and therefore experiences a state of oxidative stress should develop autism. Nothing in Dr. Deth's theoretical causal chain of events differentiates between oxidative stresses created by vaccination and oxidative stresses created by other events.

Dr. Deth also states that thimerosal reduces cellular levels of GSH in cultured neuronal cells at concentrations that are approximately 3-fold lower than plasma concentrations of mercury following administration of a single thimerosal-containing vaccine. His reference for this statement (#24) was an unpublished "manuscript in preparation." Obviously, analysis of this assertion is restricted because neither data nor methods are provided. Nevertheless, I can state that scientists recognize the limitations of in vitro work and will not extrapolate from in vitro data obtained in cultured cells to what occurs in vivo. Furthermore, Dr. Deth did not mention that this decrease in cellular GSH was associated with evidence of increased oxidative stress/damage in these cells. It is also noteworthy that, because of their artificial environment in vitro, it is difficult if not impossible to culture cells in the absence of oxidative stress and damage to the cells.

Dr. Deth relies upon the findings in a paper by Hornig et. al. 2004 (reference 26) in which neurotoxicity was observed following administration of thimerosal to different strains of mice. Dr. Deth stated on page 4 of his report that "significant neuropathy and developmental disturbances were only observed in the strain harboring genetic deficits in redox-related enzymes". <u>This is a misleading and untrue statement</u>. The strains of mice studied by Hornig et. al. were autoimmune disease-sensitive and autoimmune disease-resistant strains of mice, not mice with genetic deficits in redox-related enzymes. Regardless, Berman et al. 2008 attempted to repeat the findings of Hornig et al. and could not [13].

Dr. Deth then discusses the effects of thimerosal on GSH synthesis and methylation. These are all in vitro studies, which are difficult, if not impossible, to

extrapolate to the in vivo situation. He then revisits the effects of thimerosal on GSH production, which I have already addressed above.

Dr. Deth then hypothesizes a genetic vulnerability to thimerosal-induced toxicity, notes a number of genetic risk factors, and lists supporting references (11, 46-48). First, reference 11 has nothing to do with either thimerosal or autism. Reference 46 discusses a large number of genes involved in autism spectrum disorders, but I could not find any reference to a link between these genes and oxidative stress. Reference 47 discusses adenosine deaminase alleles and autism, but I know of no link, even theoretical, between adenosine alleles and oxidative stress.

The James et al. 2004 study (reference 9) discusses the finding of altered methionine cycle and transulfuration metabolites in autistic children [14]. The authors make the statement that the increase in GSSG and a decrease in GSH:GSSG ratio indicate oxidative stress. As I have discussed previously, I do not agree that alterations of GSH and GSSG are indicative of oxidative stress in the absence of measurements of reliable biomarkers of oxidative damage. Furthermore, James et al. did not assess or control for possible differences in dietary intake of nutrients that can alter GSH and GSSG levels. In fact, the authors make the statement in their Discussion (p. 1612) that "specific dietary differences between groups could have contributed to our results." In addition, the authors state on page 1615 that: "Our attempts to interpret these preliminary metabolic findings are clearly speculative." Finally, the authors do not relate their findings to thimerosal-containing vaccines as a cause of autism, nor would such a link be scientifically valid.

In a later study by James et al. 2006 (reference 10), the authors again observed altered GSH and GSSG levels in autistic children [15]. As in the 2004 study, the most reliable biomarkers for oxidative damage (F_2-isoprostanes as measured by gas chromatography mass spectrometery) were not obtained, so the reported biomarker abnormalities cannot be said to be conclusive. The authors also describe the finding of genetic metabolic abnormalities in some children with autism, but the differences in the frequency of these genetic alleles between children with autism and normal children were very small and most of the differences were not statistically significant. Of note is the last sentence of the paper (p. 954): "Clearly these new findings should be considered preliminary until confirmed in larger population–based studies." To my knowledge, larger population-based studies have not confirmed these results.

For these reasons, it is my opinion that the data presented in the two papers by James et. al. provide little if any convincing evidence related to the role of oxidative stress and autism.

Dr. Deth also relies heavily upon his own published experiment, Waly et al. 2004 (reference 27) [16]. This in vitro experiment reported the effect of thimerosal on insulin-like growth factor-1 and dopamine methylation in human neuroblastoma cells. It is impossible to extrapolate these findings from tumor cells to what happens in vivo. Even Dr. Deth recognizes the study's limitations, and states in the original article (p. 368) that:

"There are important limitations to our findings. We utilized a transformed cell line, and molecular events in tumor-derived cells might not mirror those in normal cells." Dr. Deth and his co-authors go on to say on p. 368: "It is obvious that biochemical studies under cultured cell conditions do not replicate the complex *in vivo* environment, in terms of ambient metal ion concentrations, redox conditions, and other factors that could influence methylation events. Further investigation of the *in vivo* and *in vitro* effects of heavy metals on growth factor-induced cellular differentiation is needed."

Despite Dr. Deth's willingness to restrict his in vitro findings in the original Waly 2004 publication, in his report filed in the current matter, Dr. Deth is willing to make significant extrapolations from its results. Indeed, the Waly paper is the foundation upon which Dr. Deth rests his causal hypothesis. The paper cannot support the extrapolations or the hypothesis, and the findings have considerable limitations already noted by the authors.

Moreover, it is worth noting that almost all cells in culture are already exposed to a significant oxidative stress because they are cultured under high concentrations of oxygen. Second, the authors discuss the potential effects of heavy metals that can catalyze oxidative chemistry. This is relevant because almost all cell media contain free iron, which is a potent catalyst of oxidative chemistry. For these reasons, the Waly 2004 findings cannot be relied upon to indicate potential in vivo effects from thimerosal-containing vaccines, nor do the findings indicate a connection between oxidative stress and autism.

Finally, Dr. Deth states that "measures that reduce oxidative stress or remove heavy metals are reported to bring clinical improvement in autism", citing references 7 and 49. The Nataf et al. 2006 authors reported some evidence for heavy metal toxicity in some children with autism as measured by excretion of certain urinary porphyrins [17]. Excretion of these porphyrins was reduced by administration of the chelator dimercaptoxuccinic acid. However, clinical improvement after chelator therapy was <u>not</u> assessed, so there is no evidence for Dr. Deth to state the children improved post chelation. Additionally, no link between urinary porphyrin levels and oxidative stress has been established. In the Boris et al. 2007 study, autistic children were treated with pioglitazone, a PPARγ agonist, which also exerts some anti-inflammatory effects [18]. Again, this study does not address the issue of oxidative stress. In addition, this study was a small study and it was not placebo controlled; as its authors stated: "the open-label nature of this study limits the ability to draw strong conclusions regarding treatment-dependent benefits."

CONCLUSION

I do not believe that Dr. Deth has presented any substantial evidence to support his claim that there is a causative link between oxidative stress and autism. Even if one accepts that there is a correlation between autism and oxidative stress, it is more likely that the oxidative stress is the result of an underlying disease process than the result of a thimerosal-containing vaccine. Dr. Deth has failed to provide reliable scientific evidence to support a claim that there is a causative link between thimerosal-containing vaccines and autism.

Signed,

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