



Peter Marks M.D., Ph.D., Director  
Center for Biologics Evaluation and Research  
The Food and Drug Administration  
10903 New Hampshire Avenue  
Silver Spring, MD 20993  
November 13, 2017

Re: Response to your letter regarding the use of mercury in prescription drugs and vaccines.

Dear Dr. Marks,

On March 30<sup>th</sup>, Robert F. Kennedy Jr. and members of the World Mercury Project met with you and your colleagues at the FDA to discuss our concerns regarding the continued use of the mercury-based preservative, thimerosal, in prescription drugs and influenza vaccines administered to pregnant women, infants and children.

During the meeting and in written letters following the meeting, we voiced concerns regarding:

- Lack of adequate safety studies prior to marketing thimerosal as a vaccine preservative.
- Thimerosal's toxicity and ineffectiveness as a preservative.
- Mercury exposure from thimerosal-containing vaccine administration resulting in mercury levels known to cause adverse outcomes.
- Exposure to vaccine-level thimerosal resulting in harmful depositions of inorganic mercury in the brain.
- The California Environmental Protection Agency's listing of all mercury-containing products as reproductive and developmental toxicants under their Proposition 65 law.

**Thimerosal was removed from all over-the-counter products when the FDA issued final rules in the [Federal Register in 1998](#) acknowledging that thimerosal is not generally recognized as being safe or effective (GRASE). Why is this same product allowed in prescription drugs and vaccines?**

At the end of our meeting, you reassured us that you would take our concerns seriously and would "follow the science" wherever it might lead you. For several months after our meeting, I contacted the FDA public liaison Ms. McNeill inquiring when we might expect to hear back from you regarding our concerns. Ms. McNeill told me that we had provided the agency with extensive information and that it was taking additional time to review the material. I was

hopeful that FDA might finally, therefore, implement the [2001 recommendation](#) of the Institute of Medicine that pregnant women, infants and children not be exposed to thimerosal-containing vaccines.

On July 11<sup>th</sup>, we received your written response to our concerns. I was dismayed by your agency's apparent unwillingness to seriously review the large archive of published science suggesting that using thimerosal is poisoning a generation of American children. From your follow-up written response, it is clear that none of the information we provided was seriously read or even minimally digested. You make it clear in your letter that you do not intend to give any serious consideration to the abundant and mushrooming evidence of thimerosal's profound toxicity. Your letter is simply an exercise in blindly promoting an incredible vaccine industry orthodoxy that is unsupportable by empirical evidence.

You cite in your written response FDA's mushy biologics regulations which define safety as "the relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time." 21CFR 600.39(p). You report that in applying this elastic regulatory standard, "FDA must weigh the risk of a vaccine or any drug against its benefits when determining whether a product is safe. If the benefits of the vaccine or other pharmaceutical product outweigh the risks of its side effects, then the FDA finds the product to be safe." You further acknowledge that "the determination of a products safety **is a relative rather than absolute measurement**", entirely subject to FDA's "discretion and expertise." Even operating under these malleable standards, FDA should consider that vaccines are products given to healthy individuals, and their risks should be measured by an extremely high bar since they are not treating a disease. Furthermore, FDA has no capacity to evaluate risks of thimerosal since, by FDA's own admission [to Congress](#), there has never been a long-term safety study performed on thimerosal in any human population including infants and pregnant women.

Vaccines containing thimerosal in the U.S. are predominantly influenza vaccines. Furthermore, thimerosal is still [widely used](#) in vaccines given to tens of millions of children in the developing world and, since U.S. policy influences worldwide policy, FDA bears responsibility for these policies. In the U.S., thimerosal-containing vaccines are administered to healthy six-month old infants, young children and pregnant women despite never having been safety tested in those populations. According to their product inserts, influenza vaccines have been associated with an increased incidence of seizures and Guillain-Barre Syndrome. Recent studies have linked influenza vaccines to [miscarriage](#), [autism](#) and, possibly, [birth defects](#). A significant percentage of influenza vaccines still contain thimerosal and studies should be done to see if thimerosal played a role in these outcomes. There has been limited testing of influenza vaccines in animal models, however, there have not been any adequate and well-controlled studies in pregnant women. Because animal studies are not always predictive of human response, the package inserts for flu vaccines reiterate that flu vaccines "should be given to a pregnant woman only if clearly needed". In addition, there are numerous VAERS reports of injuries from thimerosal-containing vaccines. Therefore, it is imperative that vaccines administered to sensitive

populations (pregnant women, infants and children) be held to the highest standards of safety. I think parents and the American public would be appalled to learn that vaccine safety determinations are “relative” and are within an FDA employee’s “discretion and expertise.” That discretion and expertise should actually require a factual basis, not just opinion. Needless to say, these decisions should be guided by the precautionary principle.

I have organized the remainder of my response into addressing the erroneous claims made in your letter.

**Your Claim: The agency evaluates whether a preservative contained in a product is at such levels that when used at the recommended dose is not toxic to the recipient and that the “FDA ... has repeatedly found that the vaccines currently being marketed that contain thimerosal are safe...”**

**WMP Response:** Please show us the data used to evaluate thimerosal safety in infants and pregnant women. We do not believe they exist.

In an [email](#) discussion regarding the use of thimerosal-containing influenza vaccines administered to pregnant women, infants and children, in 1999, Dr. William Egan, acting Director of the Center for Drugs and Biologics (CDER), recommended that the statement, “The chronic, daily ingestion reported (in several studies-primarily Seychelles study) greatly exceeds the amount of mercury that a pregnant woman would receive from a single annual dose of thimerosal-containing influenza vaccine” ***“might well be deleted.”*** Egan went on to justify his recommendation by saying that the statement ***“...in some ways is misleading. I am not sure that I would want to argue, for example, that one could take the allowed amount of mercury for a year and administer it as a bolus injection with the same outcome as having it spaced out evenly over the year: the issue then becomes one of how much of a bolus can one give at one time without harmful effect and this data does not exist (or at least I’m not aware of them).”***

Dr. Egan was right then and he is right today; such safety data do not exist. In fact, many toxicologists believe that large [bolus dose exposures](#) such as those resulting from thimerosal-containing vaccines are more harmful in comparison to small daily dose exposures that the body is much more capable of excreting without overburdening detoxification pathways in the body. This concern is supported by research that found a mercury dose given acutely may produce toxic effects, whereas the same dose distributed over a period of time may give no evidence of poisoning. ([Koos and Longo,1976](#)).

**Your Claim: “Thimerosal has a long record of safe and effective use in preventing bacterial and fungal contamination of vaccines with no ill effects other than occasional hypersensitivity and minor local reactions at the site of injection.”.**

**WMP Response:** There is ample evidence provided in multiple studies by federal agencies and independent scientists that spans the last 90 years which documents that thimerosal is neither an effective nor a safe vaccine preservative.

In a [study](#) published in the *Journal of the American Medical Association* in 1948 titled “The bacteriostatic and bactericidal actions of some mercurial compounds on hemolytic streptococci,” the authors vigorously argued that thimerosal was ineffective as a “disinfectant, germicide and antiseptic.” In the review of the literature in this paper, the authors cited eight studies from 1928, 1935, 1937, 1938, and 1944 all of which drew similar conclusions.

In 1975, the FDA convened a panel of experts to evaluate mercury-containing over-the-counter (OTC) products. The panel issued its reports in 1980 and in 1982. The FDA issued a report of the panel’s findings in the [Federal Register](#) where they concluded that “some mercury-containing preparations are not effective and others are not safe and effective for OTC topical antimicrobial use”.

With respect to thimerosal in particular, that panel found evidence from [1950](#) which concluded that “thimerosal was no better than water in protecting mice from potential fatal streptococcal infections.” Additionally, citing a [1935](#) study, the panel reported that thimerosal was “35.3 times more toxic for embryonic chick heart tissue than for *Staphylococcus aureus*.” Most of the literature reviewed addressed mercury’s lack of antibacterial properties. One [review](#) published in 1971 titled, “Three thousand years of mercury. A plea for abandonment of a dangerous, unproven therapy,” addressed mercury’s lack of effectiveness against fungal contamination as well.

The FDA-appointed expert panel concluded that “thimerosal was not safe for OTC topical use because of its potential for cell damage if applied to broken skin and its allergy potential. It is not effective as a topical antimicrobial because its bacteriostatic action can be reversed.” However, it wasn’t until 1998 that the FDA issued its [final report](#) banning the use of thimerosal in topical OTC products because it was not “safe and effective.”

There are also several more recent published reports of thimerosal’s failure as a preservative. Clusters of disease from [Group A streptococcus](#) infections were traced back to multi-dose vials of diphtheria toxoid, pertussis, and tetanus toxoid (DPT) vaccine which were contaminated after being opened. Additionally, in 2004, a Chiron plant that manufactured Fluvirin was forced to close because its vaccine was contaminated with [Serratia marcescens](#). This vaccine used thimerosal as a preservative. In this case and in the many others cited, thimerosal failed to prevent bacterial growth.

In response to the reports from the FDA expert panel who reviewed the use of thimerosal in over-the-counter products in the 1980’s, the FDA published in the April 22, 1998 Federal Register [Status of Certain Additional Over-the-Counter Drug Category II and III Active Ingredients. \(April 22, 1998\);63\(77\):19799-19802. 21 CFR Part 310 \[Docket No. 75N-183F, 75N-](#)

[183D, and 80N-0280](#) concluding that the use of thimerosal in over the counter products is not “generally recognized as safe or effective” (GRASE).

In [the final rulemaking](#), the FDA states that “safety and effectiveness have not been established for the ingredients (mercury-based preservatives) included in this current final rule and manufacturers have not submitted the necessary data in response to earlier opportunities. The agency’s experience has been that under these circumstances companies have not submitted data in response to yet another opportunity. Consumers will benefit from the early removal from the marketplace of products containing ingredients for which safety and effectiveness has not been established.”

The World Mercury Project would like to know how is it possible that one division of the FDA recognizes that **there is absolutely no safety or effectiveness data available for the use of mercury in over the counter products and essentially bans its use, while your FDA division of blood and biologics continues to recklessly allow its widespread use in [over 100 prescription products](#) including vaccines?**

**Your claim: “Under the FDA Modernization Act (FDAMA) of 1997, the FDA conducted a comprehensive review of the use of thimerosal in childhood vaccines. Conducted in 1999, this review found no evidence of harm from the use of thimerosal as a vaccine preservative, other than local hypersensitivity reactions (Ball et al. 2001).”**

**WMP Response:** It’s disturbing that according to [internal emails](#) obtained by FOIA, Dr. Ball never conducted an extensive review of reports of harm. On November 23, 1998, Dr. Leslie Ball of the FDA asked internal reviewers to perform a Medwatch query on thimerosal. Medwatch is the FDA’s database for reporting adverse drug events. On January 7, 1999, Dr. Ball was informed by Fredrick Varricchio of FDA that there were 7000 reports containing the word thimerosal on FDA’s Medwatch. He stated, “I have some results for you. Problem is that there are 7000 reports that mention thimerosal. What to do now. Obviously looking at all 7,000 is a brute force approach.” Dr. Ball responded by saying, “perhaps you can get records on a subset of 50 or so we can look at them and get a general feel for what’s been reported before we go any further.” In a subsequent email on January 19<sup>th</sup>, Mr. Varricchio noted that the “plan is to get whatever is on the summary for every [100<sup>th</sup> report](#).” This means that only 70 adverse events out of 7000 reported to the FDA were actually reviewed by Dr. Ball and her team. This email calls into question the findings reported by Dr. Ball and also suggests that an extensive investigation has never been conducted by the FDA with regard to adverse events associated with the use of thimerosal. **Would you allow any other medical product to be widely used based on review of one percent of the information available?**

I am also, Sir, frankly shocked at your unwillingness to acknowledge the robust body of literature that has been published the last 18 years since concerns regarding thimerosal first surfaced within the FDA in 1999.

There are literally hundreds of peer-reviewed, published studies that document the toxicity of thimerosal. Many of these investigated levels of mercury known to occur from vaccine exposure in cell and animal models. In [2013, Jose G. Dorea](#) published a meta-analysis of thimerosal research related to vaccine exposure. Dorea searched major databases for human and experimental studies that addressed issues related to early life exposure to TCVs. The author concluded that: “ a) mercury load in fetuses, neonates, and infants resulting from TCVs remains in blood of neonates and infants at sufficient concentration and for enough time to penetrate the brain and to exert a neurologic impact and a probable influence on neurodevelopment of susceptible infants; b) etHg metabolism related to neurodevelopmental delays has been demonstrated experimentally and observed in population studies; c) unlike chronic Hg exposure during pregnancy, neurodevelopmental effects caused by acute (repeated/cumulative) early life exposure to TCV-etHg remain unrecognized; and d) the uncertainty surrounding low-dose toxicity of etHg is challenging but recent evidence indicates that avoiding cumulative insults by alkyl-mercury forms (which include Thimerosal) is warranted.” Dorea emphasized the importance of “a) maintaining trust in vaccines while reinforcing current public health policies to abate mercury exposure in infancy; b) supporting WHO policies that recommend vaccination to prevent and control existing and impending infectious diseases; and c) not confusing the 'need' to use a specific 'product' (TCV) by accepting as 'innocuous' (or without consequences) the presence of a proven 'toxic alkyl-mercury' (etHg) at levels that have not been proven to be toxicologically safe.”

For your convenience, I have included a sampling of 35 abstracts that represent the more current state of the science regarding thimerosal that has emerged since 1999 as an appendix. Even if Dr. Ball’s review had been adequate at that time, surely 18 years of further research should prompt an updated evaluation by the FDA.

**Your Claim: A 2014 modeling study by your own Centers for Biologics Evaluation and Research employee, Dr. Robert Mitkus, showed that “peak body burdens of mercury following episodic exposures to thimerosal in this worst case did not exceed the corresponding safe body burden of mercury from MeHg at any time”.**

**WMP Response:** The [Mitkus study](#) reported that the body burden of mercury in infants, over the first 4.5 years of life following yearly exposures to thimerosal from annual flu vaccines, was two orders of magnitude lower than that estimated for exposures to the lowest regulatory threshold for MeHg over the same time period. The author relies completely on these findings to conclude that their pharmacokinetic analysis supports the safety of thimerosal when used as a preservative at current levels in certain multi-dose infant vaccines in the United States. Mitkus fails to acknowledge the past levels of exposure that infants received from vaccines starting in the late 1980s and extending well into 2000, that were 187.5 mcg etHg the first year of life versus 12.5 mcg etHg from flu vaccines annually. He also makes the assumption that there are no other mercury exposures outside of thimerosal, which is not supported by either established science or common sense.

The model developed by Mitkus relied solely on blood levels and did not take into consideration the accumulation of mercury in the brain tissue. Data from the [Burbacher](#) study that assessed exposures from both methyl and ethyl mercury in infant non-human primates, based on vaccine level exposures, found that although there was little accumulation of Hg in the blood with repeated vaccinations, accumulation of Hg in the brain of infants did occur. In fact, there was a much higher proportion of inorganic Hg in the brain of thimerosal monkeys than in the brains of MeHg monkeys (up to 71% vs. 10%). Absolute inorganic Hg concentrations in the brains of the thimerosal-exposed monkeys were approximately twice that of the MeHg monkeys. Burbacher concluded that “the safety of thimerosal drawn from blood Hg clearance data in human infants receiving vaccines may not be valid, given the significantly slower half-life of Hg in the brain as observed in the infant macaques.” But that is exactly what Mitkus does in his model and reports in his study.

Mitkus also makes the statement that thimerosal is more quickly and extensively metabolized to inorganic mercury in the brain than is MeHg and that process of dealkylation “may be” a detoxification step. According to Burbacher, who is the author of the studies relied on by Mitkus in the development of his model, the statement that dealkylation may be a detoxification process is purely speculative and has not been established. Mitkus is referring to previous reports that have indicated that dealkylation of Hg is a detoxification process that helps to protect the central nervous system ([Magos 2003](#); [Magos et al. 1985](#)). These reports are largely based on histology and histochemistry studies of adult rodents exposed to Hg for a short period of time. The results of these studies indicated that damage to the cerebellum was observed only in MeHg-treated animals that had much lower levels of inorganic Hg in the brain than animals comparably treated with ethylmercury. Moreover, the results did not indicate the presence of inorganic Hg deposits in the area where the cerebellar damage was localized (granular layer). In contrast, previous studies of adult *M. fascicularis* monkeys exposed chronically to MeHg have indicated that demethylation of Hg occurs in the brain over a long period of time after MeHg exposure and that this is not a detoxification process ([Charleston et al. 1994, 1995, 1996](#); [Vahter et al. 1994, 1995](#)). Results from these studies indicated higher inorganic Hg concentrations in the brain 6 months after MeHg exposure had ended, whereas organic Hg had cleared from the brain. The estimated half-life of organic Hg in the brain of these adult monkeys was consistent across various brain regions at approximately 37 days (similar to the brain half-life in the Burbacher study). Stereologic and autometallographic studies on the brains of these adult monkeys indicated that the persistence of inorganic Hg in the brain was associated with a significant increase in the number of microglia in the brain. ([Charleston et al. 1994, 1995, 1996](#)). The microgliosis and neuroinflammation documented in the brains of the adult monkeys in association with deposits of inorganic mercury are two hallmark findings in brain tissue of both children and adults with autism. [Neuropathological studies](#) of brain tissues from cerebellum, midfrontal, and cingulate gyrus obtained at autopsy from 11 patients with autism demonstrated the presence of an active neuroinflammatory processes in the cerebral cortex, white matter and, most notably, the cerebellum. In a subsequent study, microglia appeared markedly activated in five of 13 cases with autism, including two of three under age six, and marginally activated in an additional four of 13 cases. The [authors concluded](#) that microglial activation “represents a neuropathological alteration in a sizeable fraction of

cases with autism. Given its early presence, microglial activation may play a central role in the pathogenesis of autism in a substantial proportion of patients.”

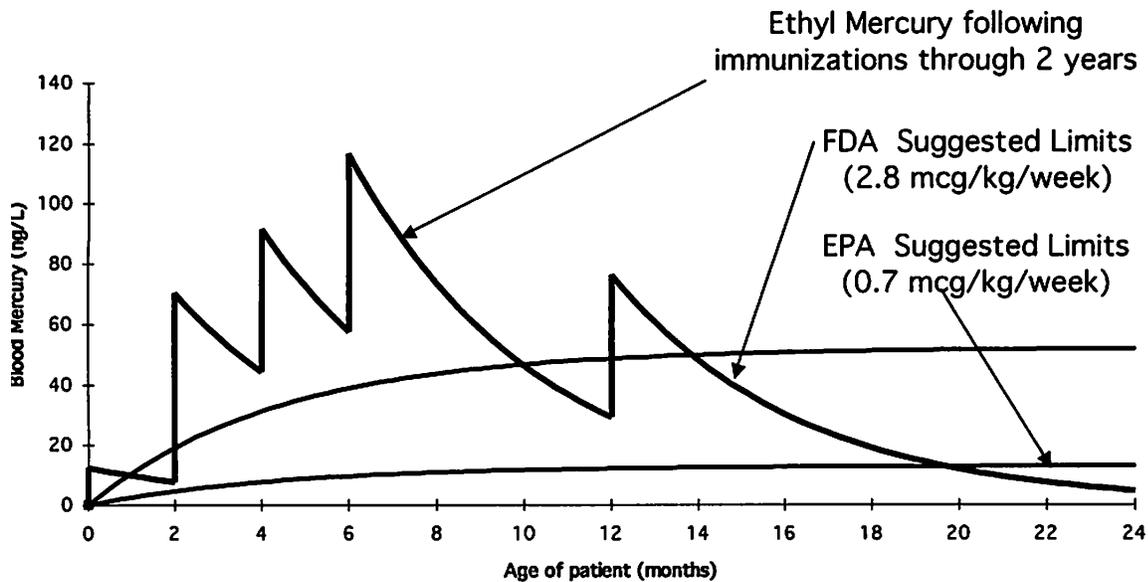
In responding to the Mitkus study, I also need to refer back to previous meetings with FDA CBER employees. When FDA assigned its pediatrician, Dr. Leslie Ball, to oversee the review, analysis and public reporting of thimerosal, Dr. Ball had little knowledge of toxicology or thimerosal. In 1999, Dr. Ball and her colleagues conducted an analysis that was prompted by the Food and Drug Modernization Act of 1997 which required FDA to compile a list of drugs and food that contain “intentionally” introduced mercury compounds and provide a qualitative and quantitative analysis of the exposure levels. They [reported](#) that the limits of exposure to mercury for an infant in the first year of life should be between 200-230 mcg total. Infants are exposed to approximately 80 to 100 mcg of organic mercury from environmental sources alone. Therefore, additional exposures from thimerosal-containing vaccines should be below 120 to 130 mcg the first year of life according to the FDA’s own findings. At the time this analysis was done, American children were routinely receiving 187.5 mcg of organic mercury during the first year of life from vaccines. This means American children were being exposed to cumulative levels of organic mercury in excess of federal safety guidelines.

The FDA consulted with an expert in the field of toxicology, Dr. Barry Rumack, MD, to better understand the potential impact of these exposure levels. Dr. Rumack had a private consulting practice where he offered “toxicologic and pharmacologic evaluation of drugs, biological and potentially toxic or hazardous agents for government and industry”. After creating several scenarios based on infants’ ages and weights, Dr. Rumack modeled both blood and body burden levels.

The [models](#) predicted sharp peaks of mercury concentrations in both blood and tissue, in a stair step sequence following each of the new thimerosal-containing vaccines given during the first six months of life. Based on these models, Rumack predicted exposure to thimerosal-containing vaccines was dosing American children with mercury levels far exceeding all three federal safety guidelines established by EPA, FDA and ATSDR. There was no point in time from birth to approximately 16-18 months of age that infants were below the EPA guidelines for allowable mercury exposure. In fact, according to the models, blood and body burden levels of mercury peaked at six months of age at a shockingly high level of 120 ng/liter. To put this in perspective, the CDC classifies mercury poisoning as blood levels of mercury greater than 10 ng/liter. What is even more concerning is that the models developed by Dr. Rumack did not take into account background exposures from environmental and dietary sources of mercury.

## Ethyl mercury kinetics after Thiomersosal injection

### vs. Methyl mercury oral suggested limits



In reporting the mercury exposure levels that result from thimerosal containing vaccines, the FDA chose not to report the findings from Rumack and Ball. Instead, they averaged the exposures over the first six months of life, even though the exposures only occurred at birth, two, four, and six months of age or during four days out of 180 days. In doing so, the agency could report that the exposures were below FDA and ATSDR guidelines in an effort to minimize concern.

In discussing this with independent toxicologists, I have been told that averaging exposures is not appropriate due to the fact that large bolus dose exposures are known to be more injurious than small daily dose exposures. If the FDA had reported the exposure levels from a daily dose perspective, it would reveal that infants were being exposed to mercury far in excess of ALL federal safety guidelines: FDA, ATSDR and EPA.

For example, my son at two months of age weighed 5 kg and received 62.5mcg Et Hg from his vaccines. According to the EPA methyl mercury guidelines of .1 mcg per kg per day, his maximum exposure level for that one day was 0.5 mcg of mercury. He received 125 times his daily allowable exposure level or 125 days of his daily allowable exposure. An analogy would be that it would be allowable to give my infant son a ½ tsp of Tylenol four times a day (320 mg), but if I gave him a 30-day dose of Tylenol (9,600 mg) on one day, it would be lethal. When I personally asked Dr. Ball why she reported the mercury exposure levels in this deceptive fashion, she responded, “That is what I was told to do.”

In a subsequent email to her superiors at FDA on July 6<sup>th</sup>, 1999 (six months after she had started her review of thimerosal), marked as being highly important and confidential and [obtained through a Freedom of Information Act request](#), Dr. Ball asked Norman Baylor, PH D, Director of the Office of Vaccines Research Review, ***“Has the application of these calculations as exposure guidelines received the sign off by toxicologists? In prior discussions, the toxicologists seemed reluctant to state any Hg (mercury) level was “safe”.*”** Although there was no response back from Dr. Baylor in the FOIA documents we received, it is obvious that the answer was no.

By 2000, there was already a mountain of evidence that thimerosal was unsafe and ineffective. For example, in 1987 the Commission of the European Communities initiated a research project on 10 known or suspected spindle poisons including thimerosal. In 1993, as described in [Mutation Research](#), 287 (1993) 17-22 thimerosal was identified as a strong inhibitor of microtubular assembly, a process which is essential for proper neuronal development. In 2000, [Stajich et al.](#) measured blood Hg levels in newborns administered the Hepatitis B vaccine, containing 12.5 mcg ethyl mercury, and found elevated post-immunization concentrations relative to pre-immunization levels in all neonates studied. Levels of blood mercury after exposure in low birth weight infants were 7.36 mcg/L ( $\pm$  4.99). One infant was found to have mercury levels of 23.6 mcg/L after exposure, which supports the inter-individual variability of mercury intoxication. The study subjects had measurable blood Hg concentrations prior to immunization, indicating that risk assessment must include background mercury levels from other sources.

I also find it disturbing that safety assessments you reference take the position that thimerosal is a necessary ingredient for influenza vaccines. This, of course, is not true. Influenza manufacturers presently make approximately two-thirds of the U.S. influenza vaccine supply without the use of thimerosal by placing the vaccine in a single dose vial or syringe, which completely eliminates the need for a preservative.

**Your Claim: The scientific evidence collected over the past 15 years does not show any evidence of harm, including serious neurodevelopmental disorders from the use of thimerosal in vaccines. The Institute of Medicine report from 2004 concluded that the evidence favors rejection of a link between thimerosal and autism based on several epidemiological studies.**

**WMP Response:** A causal relationship between autism and vaccinations cannot be proven or rejected based on evidence from population-based epidemiologic studies - period. Epidemiological studies, by definition, are not designed to prove causality; they can provide only statistical associations. Therefore, the committee’s conclusion that the “body of epidemiologic evidence favors rejection of a causal relationship...” has no scientific meaning.

Further, in the IOM [report](#) the committee admitted that population-based studies would not be able to detect subpopulations that could be genetically more vulnerable to mercury at lower doses than typical. On page 139, the report states that “This hypothesis cannot be excluded by epidemiological data from large population groups that do not show an association between a

vaccine and an adverse outcome. Depending upon the frequency of the genetic defect, a rare event caused by genetic susceptibility could be missed even in large study samples.”

What you also failed to acknowledge is that several of the same epidemiological studies reviewed by the IOM in 2004 documented an association between thimerosal-containing vaccine exposures during infancy and the subsequent development of motor and phonic tics. Tics are a family of neurological disorders that are also associated with a [diagnosis of autism](#). A significant association between Hg exposure from thimerosal-containing childhood vaccines and a diagnosis of tic disorder (TD) has now been found in six epidemiological studies ([Verstraeten et al. 2003](#), [Andrews et al. 2004](#), [Thompson et al. 2007](#), [Young et al, 2008](#), [Barile et al. 2012](#), [Geier et al. 2015](#)). The Thompson study states that, **“The replication of the findings regarding tics suggests the potential need for further studies.”** [Tozzi et al. 2009](#), also found trends towards increased motor and phonic tics with increased thimerosal exposure but these did not reach statistical significance, possibly because of the lack of a non-exposed control group. These studies employed various epidemiological methods such as case–control or cohort designs, and were conducted on cohorts of children from several different countries. In addition, several of these studies observed significant dose-dependent relationships between Hg exposure from thimerosal in vaccines and the risk of diagnosed TD. A study by [Young et al.](#) found a dose-dependent relationship between increasing Hg exposure from thimerosal in vaccines given between birth and seven months and also between birth and 13 months of age and the risk of a diagnosed TD. Researchers observed that, for a 100 µg Hg difference in exposure between birth and seven months of age, the risk for diagnosed TD was significantly increased (3.39-fold). For the same 100 µg Hg difference in exposure between birth and 13 months of age, the risk for diagnosed tics was also found to be significantly increased (4.11-fold).

Autism etiology and severity have also been associated with mercury levels. In June of this year, the international journal *Science of the Total Environment* published a compelling [study](#) from the Republic of Korea. The study identifies a strong relationship between prenatal and early childhood exposure to mercury and autistic behaviors in five-year-olds. The [MOCEH study](#) examines environmental exposures during pregnancy and childhood and their effects on children’s growth and development. A unique feature is that it includes five different blood samples: maternal blood from early and late pregnancy; cord blood; and samples from children at two and three years of age. In addition, the study asks mothers to complete three follow-up surveys and—when their child reaches age five—the 65-item Social Responsiveness Scale (SRS), which assesses autistic behaviors.

The investigators report a significant linear relationship between mercury exposure and autistic behaviors (as indicated by a scaled score called an SRS T-score). Strikingly, they find that with a doubling of blood mercury levels at four time points (late pregnancy, cord blood, and at two and three years of age), SRS T-scores are significantly higher. They also looked specifically at SRS T-scores greater than or equal to 60. Sixty and above is the accepted threshold for detecting “mild to moderate” deficits of social behavior related to autism; scores of 76 or more are in the “severe” range. In these analyses, the same linear relationship holds for late

pregnancy and birth (i.e., cord blood). With a doubling of blood mercury levels at these two time points, there is a 31% and 28% increase, respectively, in the risk of an SRS T-score of 60 or more. Finally, the researchers identify a stronger association between late-pregnancy mercury exposure and autistic behaviors in five-year-old boys versus five-year-old girls, perhaps due to mercury's endocrine-disrupting properties.

**Your Claim: Schechter and Grether, 2008, showed that California's rates of autism continued to rise while thimerosal was being phased out from three of the early childhood vaccines.**

**WMP Response:** This [study](#) has significant limitations in addressing what was really going on in the time period from 1999 to 2003. Schechter and Grether estimated exposure for each birth cohort but made no attempt to look at the actual thimerosal exposures of individual children relative to their diagnosis. In fact, looking at the data for the CDDS for the years immediately following their study, there was a notable flattening of the autism prevalence growth curve in the 2004-2006 birth cohorts, suggesting a possible effect of thimerosal phase-out. At the same time, however, any downward effect on autism rates would have been blunted by three national autism awareness campaigns, by [Autism Speaks](#), the [CDC](#) and the [AAP](#), starting early in 2005 and continuing into 2006 which raised public awareness dramatically. While thimerosal was being phased out of the Hepatitis B, Hib and DTaP vaccines over those four years, thimerosal exposure through influenza vaccines was increasing. In [2004](#), the CDC started recommending flu shots for pregnant women in any trimester. In 2004, over [90% of the supply](#) of influenza vaccines contained thimerosal. Studies of methyl mercury show that mercury is typically [1.7 times higher](#) in cord blood than in maternal blood and there are no studies investigating the pharmacokinetics of ethylmercury in pregnancy. Concurrently, in [January 2003](#), the CDC recommended flu shots with thimerosal for all children starting at six months of age. The idea that children were no longer being exposed to thimerosal was and is a fallacy.

Beyond California, in the spring of 2016, the CDC's ADDM network finally reported the autism prevalence of children born in [2004](#). For the first time that data did not show an increase in autism prevalence compared to the [2002](#) birth year cohort. They both had a one in 68 prevalence. This suggests that the removal of thimerosal from the three pediatric vaccines may have flattened autism rates prior to the widespread uptake of the flu vaccine and increased awareness. That same paper, based on children born in 2004, reported a prevalence of Autism Spectrum Disorders with IQ<70 of 4.0 per 1000. This was a 15% drop from the previous report based on children born in 2002, when the prevalence of ASDs with IQ<70 was 4.7 per 1000. Note that this had nothing to do with percentages of the ASD population or additional higher-functioning children being diagnosed – this meant that there were actually fewer severely affected children on a population basis.

Finally, your focus on autism ignores the evidence of thimerosal's associations with a range of other disorders including ADHD, speech disorders, seizure disorders, autoimmunity and eczema and the broader associations of mercury with auditory and speech impairment, nephrotoxicity and somatosensory disorders. According to the CDC, [one in six](#) American children of the

thimerosal generation now suffers from a neurodevelopmental disorder. An HHS funded study found that [54% of children](#) have a chronic disease. What evidence have you, if any, that thimerosal is not a major culprit in the epidemics that have devastated this generation? "None" is the answer!

Dr. Marks, I perceive you to be a smart man and sincere in your desire to protect children from harm. Do you, as an individual, not as the Director of CBER, really believe that the continued use of thimerosal in products given to pregnant women, infants and children, when it is completely unnecessary, is appropriate? I'm appealing to you as the mother of a young man who will never be able to take advantage of his full potential because he was harmed by thimerosal and other sources of mercury. It is my life's mission, much like the mother who started MADD, to protect all children from this completely unnecessary exposure to mercury. I ask that you please again take our concerns to heart and help support our efforts instead of regurgitating the inaccurate and indefensible positions of your agency.

Sincerely,

A handwritten signature in black ink that reads "Jim Redwood". The signature is written in a cursive style with a large, looping initial "J".

Executive Director  
World Mercury Project

## **Appendix**

### **Literature reviews**

Curr Med Chem. 2013;20(32):4060-9.

#### **[Low-dose mercury exposure in early life: relevance of thimerosal to fetuses, newborns and infants.](#)**

[Dórea JG<sup>1</sup>](#).

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This review explores the different aspects of constitutional factors in early life that modulate toxicokinetics and toxicodynamics of low-dose mercury resulting from acute ethylmercury (etHg) exposure in Thimerosal-containing vaccines (TCV). Major databases were searched for human and experimental studies that addressed issues related to early life exposure to TCV. It can be concluded that: a) mercury load in fetuses, neonates, and infants resulting from TCVs remains in blood of neonates and infants at sufficient concentration and for enough time to penetrate the brain and to exert a neurologic impact and a probable influence on neurodevelopment of susceptible infants; b) etHg metabolism related to neurodevelopmental delays has been demonstrated experimentally and observed in population studies; c) unlike chronic Hg exposure during pregnancy, neurodevelopmental effects caused by acute (repeated/cumulative) early life exposure to TCV-etHg remain unrecognized; and d) the uncertainty surrounding low-dose toxicity of etHg is challenging but recent evidence indicates that avoiding cumulative insults by alkyl-mercury forms (which include Thimerosal) is warranted. It is important to a) maintain trust in vaccines while reinforcing current public health policies to abate mercury exposure in infancy; b) generally support WHO policies that recommend vaccination to prevent and control existing and impending infectious diseases; and c) not confuse the 'need' to use a specific 'product' (TCV) by accepting as 'innocuous' (or without consequences) the presence of a proven 'toxic alkyl-mercury' (etHg) at levels that have not been proven to be toxicologically safe.

Enviro Res. 2017 Jan;152:280-293. doi: 10.1016/j.envres.2016.10.028. Epub 2016 Nov 3.

#### **[Low-dose Thimerosal in pediatric vaccines: Adverse effects in perspective.](#)**

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Vaccines are prophylactics used as the first line of intervention to prevent, control and eradicate infectious diseases. Young children (before the age of six months) are the demographic group most exposed to recommended/mandatory vaccines preserved with Thimerosal and its metabolite ethylmercury (EtHg). Particularly in the less-developed countries, newborns, neonates, and young children are exposed to EtHg because it is still in several of their pediatric vaccines and mothers are often immunized with Thimerosal-containing vaccines (TCVs) during pregnancy. While the immunogenic component of the product has undergone more rigorous testing, Thimerosal, known to have neurotoxic effects even at low doses, has not been scrutinized for the limit of tolerance alone or in combination with adjuvant-Al during immaturity or developmental periods (pregnant women, newborns, infants, and young children). Scientific evidence has shown the potential hazards of Thimerosal in experiments that modeled vaccine-EtHg concentrations. Observational population studies have revealed uncertainties related to neurological effects. However, consistently, they showed a link of EtHg with risk of certain neurodevelopment disorders, such as tic disorder, while clearly revealing the benefits of removing Thimerosal from children's vaccines (associated with immunological reactions) in developed countries. So far, only rich countries have benefited from withdrawing the risk of exposing young children to EtHg. Regarding Thimerosal administered to the very young, we have sufficient studies that characterize a state of uncertainty: the collective evidence strongly suggests that Thimerosal exposure is associated with adverse neurodevelopmental outcomes. It is claimed that the continued use of Thimerosal in the less-developed countries is due to the cost to change to another preservative, such as 2-phenoxyethanol. However, the estimated cost increase per child in the first year of life is lower than estimated lifetime cost of caring for a child with a neurodevelopmental disorder, such tic disorder. The evidence indicates that Thimerosal-free vaccine options should be made available in developing countries.

## **Animal Research**

[Neurochem Res.](#) 2012 Feb;37(2):436-47. doi: 10.1007/s11064-011-0630-z. Epub 2011 Oct 21.

### **[Administration of thimerosal to infant rats increases overflow of glutamate and aspartate in the prefrontal cortex: protective role of dehydroepiandrosterone sulfate.](#)**

[Duszczuk-Budhathoki M<sup>1</sup>](#), [Olczak M](#), [Lehner M](#), [Majewska MD](#).

Thimerosal, a mercury-containing vaccine preservative, is a suspected factor in the etiology of neurodevelopmental disorders. We previously showed that its administration to infant rats causes behavioral, neurochemical and neuropathological abnormalities similar to those present in autism. Here we examined, using microdialysis, the effect of thimerosal on extracellular levels of neuroactive amino acids in the rat prefrontal cortex (PFC). Thimerosal administration (4 injections, i.m., 240 µg Hg/kg on postnatal days 7, 9, 11, 15) induced lasting changes in amino

acid overflow: an increase of glutamate and aspartate accompanied by a decrease of glycine and alanine; measured 10-14 weeks after the injections. Four injections of thimerosal at a dose of 12.5 µg Hg/kg did not alter glutamate and aspartate concentrations at microdialysis time (but based on thimerosal pharmacokinetics, could have been effective soon after its injection). Application of thimerosal to the PFC in perfusion fluid evoked a rapid increase of glutamate overflow. Coadministration of the neurosteroid, dehydroepiandrosterone sulfate (DHEAS; 80 mg/kg; i.p.) prevented the thimerosal effect on glutamate and aspartate; the steroid alone had no influence on these amino acids. Coapplication of DHEAS with thimerosal in perfusion fluid also blocked the acute action of thimerosal on glutamate. In contrast, DHEAS alone reduced overflow of glycine and alanine, somewhat potentiating the thimerosal effect on these amino acids. Since excessive accumulation of extracellular glutamate is linked with excitotoxicity, our data imply that neonatal exposure to thimerosal-containing vaccines might induce excitotoxic brain injuries, leading to neurodevelopmental disorders. DHEAS may partially protect against mercurials-induced neurotoxicity.

[Behav Brain Res.](#) 2011 Sep 30;223(1):107-18. doi: 10.1016/j.bbr.2011.04.026. Epub 2011 Apr 28.

## [Persistent behavioral impairments and alterations of brain dopamine system after early postnatal administration of thimerosal in rats.](#)

[Olczak M<sup>1</sup>](#), [Duszczyk M](#), [Mierzejewski P](#), [Meyza K](#), [Majewska MD](#).

The neurotoxic organomercurial thimerosal (THIM), used for decades as vaccine preservative, is a suspected factor in the pathogenesis of some neurodevelopmental disorders. Previously we showed that neonatal administration of THIM at doses equivalent to those used in infant vaccines or higher, causes lasting alterations in the brain opioid system in rats. Here we investigated neonatal treatment with THIM (at doses 12, 240, 1440 and 3000 µg Hg/kg) on behaviors, which are characteristically altered in autism, such as locomotor activity, anxiety, social interactions, spatial learning, and on the brain dopaminergic system in Wistar rats of both sexes. Adult male and female rats, which were exposed to the entire range of THIM doses during the early postnatal life, manifested impairments of locomotor activity and increased anxiety/neophobia in the open field test. In animals of both sexes treated with the highest THIM dose, the frequency of prosocial interactions was reduced, while the frequency of asocial/antisocial interactions was increased in males, but decreased in females. Neonatal THIM treatment did not significantly affect spatial learning and memory. THIM-exposed rats also manifested reduced haloperidol-induced catalepsy, accompanied by a marked decline in the density of striatal D<sub>2</sub> receptors, measured by immunohistochemical staining, suggesting alterations to the brain dopaminergic system. Males were more sensitive than females to some neurodisruptive/neurotoxic actions of THIM. These data document that early postnatal THIM administration causes lasting neurobehavioral impairments and neurochemical alterations in the brain, dependent on dose and sex. If similar changes occur in THIM/mercurial-exposed children, they could contribute to neurodevelopmental disorders.

## Induction of metallothionein in mouse cerebellum and cerebrum with low-dose thimerosal injection

Minami T, Miyata E, Sakamoto Y, Yamazaki H, Ichida S. *Department of Life Sciences, School of Science & Engineering, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka, 577-8502, Japan, minamita@life.kindai.ac.jp. Cell Biol Toxicol. 2009 Apr 9.*

Thimerosal, an ethyl mercury compound, is used worldwide as a vaccine preservative. We previously observed that the mercury concentration in mouse brains did not increase with the clinical dose of thimerosal injection, but the concentration increased in the brain after the injection of thimerosal with lipopolysaccharide, even if a low dose of thimerosal was administered. Thimerosal may penetrate the brain, but is undetectable when a clinical dose of thimerosal is injected; therefore, the induction of metallothionein (MT) messenger RNA (mRNA) and protein was observed in the cerebellum and cerebrum of mice after thimerosal injection, as MT is an inducible protein. MT-1 mRNA was expressed at 6 and 9 h in both the cerebrum and cerebellum, but MT-1 mRNA expression in the cerebellum was three times higher than that in the cerebrum after the injection of 12 microg/kg thimerosal. MT-2 mRNA was not expressed until 24 h in both organs. MT-3 mRNA was expressed in the cerebellum from 6 to 15 h after the injection, but not in the cerebrum until 24 h. MT-1 and MT-3 mRNAs were expressed in the cerebellum in a dose-dependent manner. Furthermore, MT-1 protein was detected from 6 to 72 h in the cerebellum after 12 microg/kg of thimerosal was injected and peaked at 10 h. MT-2 was detected in the cerebellum only at 10 h. In the cerebrum, little MT-1 protein was detected at 10 and 24 h, and there were no peaks of MT-2 protein in the cerebrum. In conclusion, MT-1 and MT-3 mRNAs but not MT-2 mRNA are easily expressed in the cerebellum rather than in the cerebrum by the injection of low-dose thimerosal. It is thought that the cerebellum is a sensitive organ against thimerosal. As a result of the present findings, in combination with the brain pathology observed in patients diagnosed with autism, the present study helps to support the possible biological plausibility for how low-dose exposure to mercury from thimerosal-containing vaccines may be associated with autism.

## Neonatal administration of a vaccine preservative, thimerosal, produces lasting impairment of nociception and apparent activation of opioid system in rats

Olczak M, Duszczyk M, Mierzejewski P, Majewska MD. *Brain Res. 2009 Dec 8;1301:143-51. Epub 2009 Sep 9.*

Thimerosal (THIM), an organomercury preservative added to many child vaccines is a suspected factor in pathogenesis of neurodevelopmental disorders. We examined the pharmacokinetics of Hg in the brain, liver and kidneys after i.m. THIM injection in suckling rats and we tested THIM effect on nociception. THIM solutions were injected to Wistar and Lewis rats in a vaccination-like mode on PN days 7, 9, 11 and 15 in four equal doses. For Wistar rats these were: 12, 48, 240, 720, 1440, 2160, 3000 microg Hg/kg and for Lewis: 54, 216, 540 and 1080 microg Hg/kg. Pharmacokinetic analysis revealed that Hg from THIM injections accumulates in the rat brain in significant amounts and remains there longer than 30 days after the injection. At the 6th week of

age animals were examined for pain sensitivity using the hot plate test. THIM treated rats of both strains and sexes manifested statistically significantly elevated pain threshold (latency for paw licking, jumping) on a hot plate (56 degrees C). Wistar rats were more sensitive to this effect than Lewis rats. Protracted THIM-induced hypoalgesia was reversed by naloxone (5 mg/kg, i.p.) injected before the hot plate test, indicative of involvement of endogenous opioids. This was confirmed by augmented catalepsy after morphine (2.5 mg/kg, s.c.) injection. Acute THIM injection to 6-week-old rats also produced hypoalgesia, but this effect was transient and was gone within 14 days. Present findings show that THIM administration to suckling or adult rats impairs sensitivity to pain, apparently due to activation the endogenous opioid system.

### Identification and distribution of mercury species in rat tissue following administration of thimerosal or methyl mercury

*Rodrigues JL, Serpeloni JM, Batista BL, Souza SS, Barbosa F Jr. Arch Toxicol. 2010 Nov;84(11):891-6. Epub 2010 Apr 13.*

Methylmercury (Met-Hg) is one the most toxic forms of Hg, with a considerable range of harmful effects on humans. Sodium ethyl mercury thiosalicylate, thimerosal (TM) is an ethylmercury (Et-Hg)-containing preservative that has been used in manufacturing vaccines in many countries. Whereas the behavior of Met-Hg in humans is relatively well known, that of ethylmercury (Et-Hg) is poorly understood. The present study describes the distribution of mercury as (-methyl, -ethyl and inorganic mercury) in rat tissues (brain, heart, kidney and liver) and blood following administration of TM or Met-Hg. Animals received one dose/day of Met-Hg or TM by gavage (0.5 mg Hg/kg). Blood samples were collected after 6, 12, 24, 48, 96 and 120 h of exposure. After 5 days, the animals were killed, and their tissues were collected. Total blood mercury (THg) levels were determined by ICP-MS, and methylmercury (Met-Hg), ethylmercury (Et-Hg) and inorganic mercury (Ino-Hg) levels were determined by speciation analysis with LC-ICP-MS. Mercury remains longer in the blood of rats treated with Met-Hg compared to that of TM-exposed rats. Moreover, after 48 h of the TM treatment, most of the Hg found in blood was inorganic. Of the total mercury found in the brain after TM exposure, 63% was in the form of Ino-Hg, with 13.5% as Et-Hg and 23.7% as Met-Hg. In general, mercury in tissues and blood following TM treatment was predominantly found as Ino-Hg, but a considerable amount of Et-Hg was also found in the liver and brain. Taken together, our data demonstrated that the toxicokinetics of TM is completely different from that of Met-Hg. Thus, Met-Hg is not an appropriate reference for assessing the risk from exposure to TM-derived Hg. It also adds new data for further studies in the evaluation of TM toxicity.

### Gender-selective toxicity of thimerosal

*Branch DR Exp Toxicol Pathol. 2008 Sep 2. [Epub ahead of print]*

A recent report shows a correlation of the historical use of thimerosal in therapeutic immunizations with the subsequent development of autism; however, this association remains controversial. Autism occurs approximately four times more frequently in males compared to females; thus, studies of thimerosal toxicity should take into consideration gender-selective

effects. The present study was originally undertaken to determine the maximum tolerated dose (MTD) of thimerosal in male and female CD1 mice. However, during the limited MTD studies, it became apparent that thimerosal has a differential MTD that depends on whether the mouse is male or female. At doses of 38.4-76.8mg/kg using 10% DMSO as diluent, seven of seven male mice compared to zero of seven female mice tested succumbed to thimerosal. Although the thimerosal levels used were very high, as we were originally only trying to determine MTD, it was completely unexpected to observe a difference of the MTD between male and female mice. Thus, our studies, although not directly addressing the controversy surrounding thimerosal and autism, and still preliminary due to small numbers of mice examined, provide, nevertheless, the first report of gender-selective toxicity of thimerosal and indicate that any future studies of thimerosal toxicity should take into consideration gender-specific differences.

Cerebellum. 2012 Jun;11(2):575-86. doi: 10.1007/s12311-011-0319-5.

### [Maternal thimerosal exposure results in aberrant cerebellar oxidative stress, thyroid hormone metabolism, and motor behavior in rat pups; sex- and strain-dependent effects.](#)

[Sulkowski ZL](#)<sup>1</sup>, [Chen T](#), [Midha S](#), [Zavacki AM](#), [Sajdel-Sulkowska EM](#).

Methylmercury (Met-Hg) and ethylmercury (Et-Hg) are powerful toxicants with a range of harmful neurological effects in humans and animals. While Met-Hg is a recognized trigger of oxidative stress and an endocrine disruptor impacting neurodevelopment, the developmental neurotoxicity of Et-Hg, a metabolite of thimerosal (TM), has not been explored. We hypothesized that TM exposure during the perinatal period impairs central nervous system development, and specifically the cerebellum, by the mechanism involving oxidative stress. To test this, spontaneously hypertensive rats (SHR) or Sprague-Dawley (SD) rat dams were exposed to TM (200 µg/kg body weight) during pregnancy (G10-G15) and lactation (P5-P10). Male and female neonates were evaluated for auditory and motor function; cerebella were analyzed for oxidative stress and thyroid metabolism. TM exposure resulted in a delayed startle response in SD neonates and decreased motor learning in SHR male (22.6%), in SD male (29.8%), and in SD female (55.0%) neonates. TM exposure also resulted in a significant increase in cerebellar levels of the oxidative stress marker 3-nitrotyrosine in SHR female (35.1%) and SD male (14.0%) neonates. The activity of cerebellar type 2 deiodinase, responsible for local intra-brain conversion of thyroxine to the active hormone, 3',3,5-triiodothyronine (T3), was significantly decreased in TM-exposed SHR male (60.9%) pups. This coincided with an increased (47.0%) expression of a gene negatively regulated by T3, *Odf4* suggesting local intracerebellar T3 deficiency. Our data thus demonstrate a negative neurodevelopmental impact of perinatal TM exposure which appears to be both strain- and sex-dependent.

Neurochem Res. 2011 Jun;36(6):927-38. doi: 10.1007/s11064-011-0427-0. Epub 2011 Feb 25.

## [Integrating experimental \(in vitro and in vivo\) neurotoxicity studies of low-dose thimerosal relevant to vaccines.](#)

[Dórea JG](#)<sup>1</sup>.

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There is a need to interpret neurotoxic studies to help deal with uncertainties surrounding pregnant mothers, newborns and young children who must receive repeated doses of Thimerosal-containing vaccines (TCVs). This review integrates information derived from emerging experimental studies (in vitro and in vivo) of low-dose Thimerosal (sodium ethyl mercury thiosalicylate). Major databases (PubMed and Web-of-science) were searched for in vitro and in vivo experimental studies that addressed the effects of low-dose Thimerosal (or ethylmercury) on neural tissues and animal behaviour. Information extracted from studies indicates that: (a) activity of low doses of Thimerosal against isolated human and animal brain cells was found in all studies and is consistent with Hg neurotoxicity; (b) the neurotoxic effect of ethylmercury has not been studied with co-occurring adjuvant-Al in TCVs; (c) animal studies have shown that exposure to Thimerosal-Hg can lead to accumulation of inorganic Hg in brain, and that (d) doses relevant to TCV exposure possess the potential to affect human neuro-development. Thimerosal at concentrations relevant for infants' exposure (in vaccines) is toxic to cultured human-brain cells and to laboratory animals. The persisting use of TCV (in developing countries) is counterintuitive to global efforts to lower Hg exposure and to ban Hg in medical products; its continued use in TCV requires evaluation of a sufficiently nontoxic level of ethylmercury compatible with repeated exposure (co-occurring with adjuvant-Al) during early life.

## **Cellular Research**

Hum Vaccin Immunother. 2014;10(8):2328-35. doi: 10.4161/hv.29520.

## [Thimerosal compromises human dendritic cell maturation, IL-12 production, chemokine release, and T-helper polarization.](#)

[Loison E](#)<sup>1</sup>, [Gougeon ML](#).

Thimerosal is a preservative used in multidose vials of vaccine formulations to prevent bacterial and fungal contamination. We recently reported that nanomolar concentrations of thimerosal induce cell cycle arrest of human T cells activated via the TCR and inhibition of proinflammatory cytokine production, thus interfering with T-cell functions. Given the essential role of dendritic cells (DCs) in T-cell polarization and vaccine immunity, we studied the influence of non-toxic concentrations of thimerosal on DC maturation and functions. Ex-vivo exposure of human monocyte-derived DCs to nanomolar concentrations of thimerosal prevented LPS-induced DC maturation, as evidenced by the inhibition of morphological changes and a

decreased expression of the maturation markers CD86 and HLA-DR. In addition, thimerosal dampened their proinflammatory response, in particular the production of the Th1 polarizing cytokine IL-12, as well as TNF- $\alpha$  and IL-6. DC-dependent T helper polarization was altered, leading to a decreased production of IFN- $\gamma$  IP10 and GM-CSF and increased levels of IL-8, IL-9, and MIP-1 $\alpha$ . Although multi-dose vials of vaccines containing thimerosal remain important for vaccine delivery, our results alert about the ex-vivo immunomodulatory effects of thimerosal on DCs, a key player for the induction of an adaptive response.

J Toxicol. 2013;2013:801517. doi: 10.1155/2013/801517. Epub 2013 Jun 9.

### [B-lymphocytes from a population of children with autism spectrum disorder and their unaffected siblings exhibit hypersensitivity to thimerosal.](#)

[Sharpe MA](#)<sup>1</sup>, [Gist TL](#), [Baskin DS](#).

The role of thimerosal containing vaccines in the development of autism spectrum disorder (ASD) has been an area of intense debate, as has the presence of mercury dental amalgams and fish ingestion by pregnant mothers. We studied the effects of thimerosal on cell proliferation and mitochondrial function from B-lymphocytes taken from individuals with autism, their nonautistic twins, and their nontwin siblings. Eleven families were examined and compared to matched controls. B-cells were grown with increasing levels of thimerosal, and various assays (LDH, XTT, DCFH, etc.) were performed to examine the effects on cellular proliferation and mitochondrial function. A subpopulation of eight individuals (4 ASD, 2 twins, and 2 siblings) from four of the families showed thimerosal hypersensitivity, whereas none of the control individuals displayed this response. The thimerosal concentration required to inhibit cell proliferation in these individuals was only 40% of controls. Cells hypersensitive to thimerosal also had higher levels of oxidative stress markers, protein carbonyls, and oxidant generation. This suggests certain individuals with a mild mitochondrial defect may be highly susceptible to mitochondrial specific toxins like the vaccine preservative thimerosal.

Toxicol Appl Pharmacol. 2015 Aug 1;286(3):216-23. doi: 10.1016/j.taap.2015.05.002. Epub 2015 May 14.

### [Toxicological effects of thiomersal and ethylmercury: Inhibition of the thioredoxin system and NADP\(+\)-dependent dehydrogenases of the pentose phosphate pathway.](#)

[Rodrigues J](#)<sup>1</sup>, [Branco V](#)<sup>2</sup>, [Lu J](#)<sup>3</sup>, [Holmgren A](#)<sup>3</sup>, [Carvalho C](#)<sup>4</sup>.

Mercury (Hg) is a strong toxicant affecting mainly the central nervous, renal, cardiovascular and immune systems. Thiomersal (TM) is still in use in medical practice as a topical antiseptic and as

a preservative in multiple dose vaccines, routinely given to young children in some developing countries, while other forms of mercury such as methylmercury represent an environmental and food hazard. The aim of the present study was to determine the effects of thiomersal (TM) and its breakdown product ethylmercury (EtHg) on the thioredoxin system and NADP(+)-dependent dehydrogenases of the pentose phosphate pathway. Results show that TM and EtHg inhibited the thioredoxin system enzymes in purified suspensions, being EtHg comparable to methylmercury (MeHg). Also, treatment of neuroblastoma and liver cells with TM or EtHg decreased cell viability (GI50: 1.5 to 20 $\mu$ M) and caused a significant ( $p < 0.05$ ) decrease in the overall activities of thioredoxin (Trx) and thioredoxin reductase (TrxR) in a concentration- and time-dependent manner in cell lysates. Compared to control, the activities of Trx and TrxR in neuroblastoma cells after EtHg incubation were reduced up to 60% and 80% respectively, whereas in hepatoma cells the reduction was almost 100%. In addition, the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also significantly inhibited by all mercurials, with inhibition intensity of  $\text{Hg}(2+) > \text{MeHg} \approx \text{EtHg} > \text{TM}$  ( $p < 0.05$ ). Cell incubation with sodium selenite alleviated the inhibitory effects on TrxR and glucose-6-phosphate dehydrogenase. Thus, the molecular mechanism of toxicity of TM and especially of its metabolite EtHg encompasses the blockage of the electrons from NADPH via the thioredoxin system.

## [Biochemical and molecular basis of thimerosal-induced apoptosis in T cells: A major role of mitochondrial pathway](#)

*Makani S, Gollapudi S, Yel L, Chiplunkar S, Gupta S. Genes & Immunity. 2002 Aug;3(5):270-8.*

The major source of thimerosal (ethyl mercury thiosalicylate) exposure is childhood vaccines. It is believed that the children are exposed to significant accumulative dosage of thimerosal during the first 2 years of life via immunization. Because of health-related concerns for exposure to mercury, we examined the effects of thimerosal on the biochemical and molecular steps of mitochondrial pathway of apoptosis in Jurkat T cells. Thimerosal and not thiosalicylic acid (non-mercury component of thimerosal), in a concentration-dependent manner, induced apoptosis in T cells as determined by TUNEL and propidium iodide assays, suggesting a role of mercury in T cell apoptosis. Apoptosis was associated with depolarization of mitochondrial membrane, release of cytochrome c and apoptosis inducing factor (AIF) from the mitochondria, and activation of caspase-9 and caspase-3, but not of caspase-8. In addition, thimerosal in a concentration-dependent manner inhibited the expression of XIAP, cIAP-1 but did not influence cIAP-2 expression. Furthermore, thimerosal enhanced intracellular reactive oxygen species and reduced intracellular glutathione (GSH). Finally, exogenous glutathione protected T cells from thimerosal-induced apoptosis by upregulation of XIAP and cIAP1 and by inhibiting activation of both caspase-9 and caspase-3. These data suggest that thimerosal induces apoptosis in T cells via mitochondrial pathway by inducing oxidative stress and depletion of GSH.

WMP Note: This study found that even micromolar concentrations of thimerosal can cause apoptosis.

## Thimerosal induces micronuclei in the cytochalasin B block micronucleus test with human lymphocytes

Westphal GA, Asgari S, Schulz TG, Bünger J, Müller M, Hallier E. *Archives of Toxicology*. 2003 Jan; 77(1):50 – 55.

Significant induction of micronuclei was seen at concentrations of thimerosal between 0.05-0.5 µg/ml in 14 out of 16 experiments. Thus, genotoxic effects were seen even at concentrations which can occur at the injection site. Toxicity and toxicity-related elevation of micronuclei was seen at and above 0.6 µg/ml thimerosal. Marked individual and intraindividual variations in the in vitro response to thimerosal among the different blood donors occurred. However, there was no association observed with any of the glutathione S-transferase polymorphism investigated. In conclusion, thimerosal is genotoxic in the cytochalasin B block micronucleus test with human lymphocytes (immune cells). These data raise some concern on the widespread use of thimerosal.

## Thimerosal induces DNA breaks, Caspase-3 activation, membrane damage, and cell death in cultured human neurons and fibroblasts

Baskin DS, Ngo H, Didenko VV. *Toxicological Sciences*. 2003 Aug;74(2):361-8.

Thimerosal is an organic mercurial compound used as a preservative in biomedical preparations. Little is known about the reactions of human neuronal and skin cells to its micro- and nanomolar concentrations, which can occur after using thimerosal-containing products. A useful combination of fluorescent techniques for the assessment of thimerosal toxicity is introduced. Short-term thimerosal toxicity was investigated in cultured human cerebral cortical neurons and in normal human fibroblasts. Cells were incubated with 125-nM to 250-µM concentrations of thimerosal for 45 min to 24 h. A 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) dye exclusion test was used to identify nonviable cells and terminal transferase-based nick-end labeling (TUNEL) to label DNA damage. Detection of active caspase-3 was performed in live cell cultures using a cell-permeable fluorescent caspase inhibitor. The morphology of fluorescently labeled nuclei was analyzed. After 6 h of incubation, the thimerosal toxicity was observed at 2 µM based on the manual detection of the fluorescent attached cells and at a 1-µM level with the more sensitive GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence. The lower limit did not change after 24 h of incubation. Cortical neurons demonstrated higher sensitivity to thimerosal compared to fibroblasts. The first sign of toxicity was an increase in membrane permeability to DAPI after 2 h of incubation with 250 µM thimerosal. A 6-h incubation resulted in failure to exclude DAPI, generation of DNA breaks, caspase-3 activation, and development of morphological signs of apoptosis. We demonstrate that thimerosal in micromolar concentrations rapidly induce membrane and DNA damage and initiate

caspase-3–dependent apoptosis in human neurons and fibroblasts. We conclude that a proposed combination of fluorescent techniques can be useful in analyzing the toxicity of thimerosal.

WMP Note: Baskin documented that thimerosal disrupts cell membranes, damages DNA and alters cell shape at concentrations only 4 times those expected from vaccines.

### [Activation of methionine synthase by insulin-like growth factor-1 and dopamine: a target for neurodevelopmental toxins and thimerosal.](#)

*Waly M, Olteanu H, Banerjee R, Choi SW, Mason JB, Parker BS, Sukumar S, Shim S, Sharma A, Benzecry JM, Power-Charnitsky VA, Deth RC. Mol Psychiatry. 2004 Apr;9(4):358-70.*

Methylation events play a critical role in the ability of growth factors to promote normal development. Neurodevelopmental toxins, such as ethanol and heavy metals, interrupt growth factor signaling, raising the possibility that they might exert adverse effects on methylation. We found that insulin-like growth factor-1 (IGF-1)- and dopamine-stimulated methionine synthase (MS) activity and folate-dependent methylation of phospholipids in SH-SY5Y human neuroblastoma cells, via a PI3-kinase- and MAP-kinase-dependent mechanism. The stimulation of this pathway increased DNA methylation, while its inhibition increased methylation-sensitive gene expression. Ethanol potently interfered with IGF-1 activation of MS and blocked its effect on DNA methylation, whereas it did not inhibit the effects of dopamine. Metal ions potently affected IGF-1 and dopamine-stimulated MS activity, as well as folate-dependent phospholipid methylation: Cu(2+) promoted enzyme activity and methylation, while Cu(+), Pb(2+), Hg(2+) and Al(3+) were inhibitory. The ethylmercury-containing preservative thimerosal inhibited both IGF-1- and dopamine-stimulated methylation with an IC(50) of 1 nM and eliminated MS activity. Our findings outline a novel growth factor signaling pathway that regulates MS activity and thereby modulates methylation reactions, including DNA methylation. The potent inhibition of this pathway by ethanol, lead, mercury, aluminum and thimerosal suggests that it may be an important target of neurodevelopmental toxins.

### [Uncoupling of ATP-mediated calcium signaling and dysregulation interleukin-6 secretion in dendritic cells by nanomolar thimerosal](#)

*Goth SR, Chu RA, Gregg JP, Cherednichenko G, Pessah IN. Environ Health Perspect. 2006 Jul;114(7):1083-91.*

Dendritic cells (DCs), a rare cell type widely distributed in the soma, are potent antigen-presenting cells that initiate primary immune responses. DCs rely on intracellular redox state and calcium (Ca<sup>2+</sup>) signals for proper development and function, but the relationship between these two signaling systems is unclear. Thimerosal (THI) is a mercurial used to preserve vaccines and consumer products, and is used experimentally to induce Ca<sup>2+</sup> release from microsomal stores. We tested adenosine triphosphate (ATP)-mediated Ca<sup>2+</sup> responses of DCs transiently

exposed to nanomolar THI. Transcriptional and immunocytochemical analyses show that murine myeloid immature DCs (IDCs) and mature DCs (MDCs) express inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) and ryanodine receptor (RyR) Ca<sup>2+</sup> channels, known targets of THI. IDCs express the RyR1 isoform in a punctate distribution that is densest near plasma membranes and within dendritic processes, whereas IP<sub>3</sub>Rs are more generally distributed. RyR1 positively and negatively regulates purinergic signaling because ryanodine (Ry) blockade *a*) recruited 80% more ATP responders, *b*) shortened ATP-mediated Ca<sup>2+</sup> transients > 2-fold, and *c*) produced a delayed and persistent rise (≥ 2-fold) in baseline Ca<sup>2+</sup>. THI (100 nM, 5 min) recruited more ATP responders, shortened the ATP-mediated Ca<sup>2+</sup> transient (≥ 1.4-fold), and produced a delayed rise (≥ 3-fold) in the Ca<sup>2+</sup> baseline, mimicking Ry. THI and Ry, in combination, produced additive effects leading to uncoupling of IP<sub>3</sub>R and RyR1 signals. THI altered ATP-mediated interleukin-6 secretion, initially enhancing the rate of cytokine secretion but suppressing cytokine secretion overall in DCs. DCs are exquisitely sensitive to THI, with one mechanism involving the uncoupling of positive and negative regulation of Ca<sup>2+</sup> signals contributed by RyR1.

### [Thimerosal induces neuronal cell death by causing cytochrome C and apoptosis-inducing factor release from mitochondria](#)

*Yel L, Brown LE, Su K, Gollapudi S, Gupta S. Int J Mol Med. 2005 Dec;16(6):971-7.*

There is a worldwide increasing concern over the neurological risks of thimerosal (ethylmercury thiosalicylate) which is an organic mercury compound that is commonly used as an antimicrobial preservative. In this study, we show that thimerosal, at nanomolar concentrations, induces neuronal cell death through the mitochondrial pathway. Thimerosal, in a concentration- and time-dependent manner, decreased cell viability as assessed by calcein-ethidium staining and caused apoptosis detected by Hoechst 33258 dye. Thimerosal-induced apoptosis was associated with depolarization of mitochondrial membrane, generation of reactive oxygen species, and release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria to cytosol. Although thimerosal did not affect cellular expression of Bax at the protein level, we observed translocation of Bax from cytosol to mitochondria. Finally, caspase-9 and caspase-3 were activated in the absence of caspase-8 activation. Our data suggest that thimerosal causes apoptosis in neuroblastoma cells by changing the mitochondrial microenvironment.

### [In vitro uptake of glutamate in glast and GLT-1 transfected mutant CHO-K1 cells is inhibited by the ethylmercury-containing preservative thimerosal](#)

*Mutkus L, Aschner JL, Syversen T, Shanker G, Sonnewald U, Aschner M. Bio Trace Elem Res. 2005 Summer;105(1-3):71-86*

Thimerosal, also known as thimersal, Merthrolate, or sodiummethyl-mercurithiosalicylate, is an organic mercurial compound that is used in a variety of commercial as well as biomedical applications. As a preservative, it is used in a number of vaccines and pharmaceutical products. Its active ingredient is ethylmercury. Both inorganic and organic mercurials are known to interfere with glutamate homeostasis. Brain glutamate is removed mainly by astrocytes from the extracellular fluid via high-affinity astroglial Na<sup>+</sup>-dependent excitatory amino acid transporters, glutamate/ aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1). The effects of thimerosal on glutamate homeostasis have yet to be determined. As a first step in this process, we examined the effects of thimerosal on the transport of [<sup>3</sup>H]-D-aspartate, a nonmetabolizable glutamate analog, in Chinese hamster ovary (CHO) cells transfected with two glutamate transporter subtypes, GLAST (EAAT1) and GLT-1 (EAAT2). Additionally, studies were undertaken to determine the effects of thimerosal on mRNA and protein levels of these transporters. The results indicate that thimerosal treatment caused significant but selective changes in both glutamate transporter mRNA and protein expression in CHO cells. Thimerosal-mediated inhibition of glutamate transport in the CHO-K1 cell line DdB7 was more pronounced in the GLT-1-transfected cells compared with the GLAST-transfected cells. These studies suggest that thimerosal accumulation in the central nervous system might contribute to dysregulation of glutamate homeostasis.

WMP Note: Glutamate is a neurotransmitter necessary for proper brain functioning. Yip (2007) documented decreased levels of glutamate in autistic cerebral brain tissue and Hornig (2004) noted altered glutamate receptors in thimerosal exposed mice.

### [Thimerosal neurotoxicity is associated with glutathione depletion: protection with glutathione precursors](#)

JAMES SJ, SLIKKER W 3RD, MELNYK S, NEW E, POGRIBNA M, JERNIGAN S. *NEUROTOXICOLOGY*. 2005 JAN;26(1):1-8.

Thimerosal is an antiseptic containing 49.5% ethyl mercury that has been used for years as a preservative in many infant vaccines and in flu vaccines. Environmental methyl mercury has been shown to be highly neurotoxic, especially to the developing brain. Because mercury has a high affinity for thiol (sulfhydryl (—SH)) groups, the thiol-containing antioxidant, glutathione (GSH), provides the major intracellular defense against mercury-induced neurotoxicity. Cultured neuroblastoma cells were found to have lower levels of GSH and increased sensitivity to thimerosal toxicity compared to glioblastoma cells that have higher basal levels of intracellular GSH. Thimerosal-induced cytotoxicity was associated with depletion of intracellular GSH in both cell lines. Pretreatment with 100 μM glutathione ethyl ester or N-acetylcysteine (NAC), but not methionine, resulted in a significant increase in intracellular GSH in both cell types. Further, pretreatment of the cells with glutathione ethyl ester or NAC prevented cytotoxicity with exposure to 15 μM Thimerosal. Although Thimerosal has been recently removed from most children's vaccines, it is still present in flu vaccines given to pregnant women, the elderly, and to children in developing countries. The potential protective effect of GSH or NAC against mercury toxicity warrants further research as possible adjunct therapy to individuals still receiving Thimerosal-containing vaccinations.

WMP Note: James notes that the viability of neuronal cell lines was decreased after just 3-hour exposure to 2.5 micromolar concentrations of thimerosal

## [Thimerosal induces apoptosis in a neuroblastoma model via the CJUN-N-terminal kinase pathway](#)

*Herdman ML, Marcelo A, Huang Y, Niles RM, Dhar S, Kinningham KK. Toxicol Sci. 2006 Jul;92(1):246-53.*

The cJun N-terminal kinase (JNK)-signaling pathway is activated in response to a variety of stimuli, including environmental insults, and has been implicated in neuronal apoptosis. In this study, we investigated the role that the JNK pathway plays in neurotoxicity caused by thimerosal, an ethylmercury-containing preservative. SK-N-SH cells treated with thimerosal (0–10 $\mu$ M) showed an increase in the phosphorylated (active) form of JNK and cJun with 5 and 10 $\mu$ M thimerosal treatment at 2 and 4 h. To examine activator protein-1 (AP-1) transcription, cells were transfected with a pGL2 vector containing four AP-1 consensus sequences and then treated with thimerosal (0–2.5 $\mu$ M) for 24 h. Luciferase studies showed an increase in AP-1 transcriptional activity upon thimerosal administration. To determine the components of the AP-1 complex, cells were transfected with a dominant negative to either cFos (A-Fos) or cJun (TAM67). Reporter analysis showed that TAM67, but not A-Fos, decreased AP-1 transcriptional activity, indicating a role for cJun in this pathway. To assess which components are essential to apoptosis, cells were treated with a cell-permeable JNK inhibitor II (SP600125) or transfected with TAM67, and the downstream effectors of apoptosis were analyzed. Cells pretreated with SP600125 showed decreases in activation of caspases 9 and 3, decreases in degradation of poly(ADP-ribose) polymerase (PARP), and decreased levels of proapoptotic Bim, in comparison to cells treated with thimerosal alone. However, cells transfected with TAM67 showed no changes in those same components. Taken together, these results indicate that thimerosal-induced neurotoxicity occurs through the JNK-signaling pathway, independent of cJun activation, leading ultimately to apoptotic cell death.

## [Thimerosal induces TH2 responses via influencing cytokine secretion by human dendritic cells](#)

*Agrawal A, Kaushal P, Agrawal S, Gollapudi S, Gupta S. J Leukoc Biol. 2007 Feb;81(2):474-82.*

Thimerosal is an organic mercury compound that is used as a preservative in vaccines and pharmaceutical products. Recent studies have shown a TH2-skewing effect of mercury, although the underlying mechanisms have not been identified. In this study, we investigated whether thimerosal can exercise a TH2-promoting effect through modulation of functions of dendritic cells (DC). Thimerosal, in a concentration-dependent manner, inhibited the secretion of LPS-induced proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p70 from human monocyte-derived DC. However, the secretion of IL-10 from DC was not affected. These thimerosal-exposed DC induced increased TH2 (IL-5 and IL-13) and decreased TH1 (IFN- $\gamma$ ) cytokine secretion from the

T cells in the absence of additional thimerosal added to the coculture. Thimerosal exposure of DC led to the depletion of intracellular glutathione (GSH), and addition of exogenous GSH to DC abolished the TH2-promoting effect of thimerosal-treated DC, restoring secretion of TNF- $\alpha$ , IL-6, and IL-12p70 by DC and IFN- $\gamma$  secretion by T cells. These data suggest that modulation of TH2 responses by mercury and thimerosal, in particular, is through depletion of GSH in DC.

WMP Note: James has documented that children with autism have low levels of plasma glutathione.

## [Effects of thimerosal on NGF signal transduction and cell death in neuroblastoma cells](#)

*Parran DK, Barker A, Ehrich M. Toxicological Sciences. 2005 Jul;86(1):132-40.*

Signaling through neurotrophic receptors is necessary for differentiation and survival of the developing nervous system. The present study examined the effects of the organic mercury compound thimerosal on nerve growth factor signal transduction and cell death in a human neuroblastoma cell line (SH-SY5Y cells). Following exposure to 100 ng/ml NGF and increasing concentrations of thimerosal (1 nM–10  $\mu$ M), we measured the activation of TrkA, MAPK, and PKC- $\delta$ . In controls, the activation of TrkA MAPK and PKC- $\delta$  peaked after 5 min of exposure to NGF and then decreased but was still detectable at 60 min. Concurrent exposure to increasing concentrations of thimerosal and NGF for 5 min resulted in a concentration-dependent decrease in TrkA and MAPK phosphorylation, which was evident at 50 nM for TrkA and 100 nM for MAPK. Cell viability was assessed by the LDH assay. Following 24-h exposure to increasing concentrations of thimerosal, the EC50 for cell death in the presence or absence of NGF was 596 nM and 38.7 nM, respectively. Following 48-h exposure to increasing concentrations of thimerosal, the EC50 for cell death in the presence and absence of NGF was 105 nM and 4.35 nM, respectively. This suggests that NGF provides protection against thimerosal cytotoxicity. To determine if apoptotic versus necrotic cell death was occurring, oligonucleosomal fragmented DNA was quantified by ELISA. Control levels of fragmented DNA were similar in both the presence and absence of NGF. With and without NGF, thimerosal caused elevated levels of fragmented DNA appearing at 0.01  $\mu$ M (apoptosis) to decrease at concentrations  $>1$   $\mu$ M (necrosis). These data demonstrate that thimerosal could alter NGF-induced signaling in neurotrophin-treated cells at concentrations lower than those responsible for cell death.

## [Genotoxicity of thimerosal in cultured human lymphocytes with and without metabolic activation sister chromatid exchange analysis proliferation index and mitotic index](#)

*Eke D, Celik A. Mersin University, Faculty of Science and Letters, Department of Biology, 33343 Mersin, Turkey. Toxicol In Vitro. 2008 Jun;22(4):927-34. Epub 2008 Feb 1.*

Thimerosal is an antiseptic containing 49.5% of ethyl mercury that has been used for years as a preservative in many infant vaccines and in flu vaccines. Thimerosal is an organic mercurial compound used as a preservative in biomedical preparations. In this study, we evaluated the genotoxic effect of thimerosal in cultured human peripheral blood lymphocytes using sister chromatid exchange analysis in culture conditions with and without S9 metabolic activation. This study is the first report investigating the genotoxic effects of thimerosal in cultured human peripheral blood lymphocyte cells using sister chromatid exchange analysis. An analysis of variance test (ANOVA) was performed to evaluate the results. Significant induction of sister chromatid exchanges was seen at concentrations between 0.2 and 0.6 microg/ml of thimerosal compared with negative control. A significant decrease ( $p < 0.001$ ) in mitotic index (MI) and proliferation index (PRI) as well as an increase in SCE frequency ( $p < 0.001$ ) was observed compared with control cultures. Our results indicate the genotoxic and cytotoxic effect of TH in cultured human peripheral blood lymphocytes at tested doses in cultures with/without S9 fraction.

### [Characterization of early events involved in human dendritic cell maturation induced by sensitizers: cross talk between mapk signalling pathways](#)

*Trompezinski S, Migdal C, Tailhardat M, Le Varlet B, Courtellemont P, Haftek M, Serres M. Toxicol Appl Pharmacol. 2008 Aug 1;230(3):397-406. Epub 2008 Apr 8.*

Dendritic cells (DCs), efficient-antigen presenting cells play an important role in initiating and regulating immune responses. DC maturation following exposure to nickel or DNCB induced an up-regulation of phenotypic markers and inflammatory cytokine secretion. Early intracellular mechanisms involved in DC maturation required to be precise. To address this purpose, DCs derived from human monocytes were treated with sensitizers (nickel, DNCB or thimerosal) in comparison with an irritant (SDS). Our data confirming the up-regulation of CD86, CD54 and cytokine secretion (IL-8 and TNF $\alpha$ ) induced by sensitizers but not by SDS, signalling transduction involved in DC maturation was investigated using these chemicals. Kinase activity measurement was assessed using two new sensitive procedures (Facetrade mark and CBA) requiring few cells. SDS did not induce changes in signalling pathways whereas NiSO $_4$ , DNCB and thimerosal markedly activated p38 MAPK and JNK, in contrast Erk1/2 phosphorylation was completely inhibited by DNCB or thimerosal and only activated by nickel. A pre-treatment with p38 MAPK inhibitor (SB203580) suppressed Erk1/2 inhibition induced by DNCB or thimerosal demonstrating a direct interaction between p38 MAPK and Erk1/2. A pre-treatment with an antioxidant, N-acetyl-L-cysteine (NAC) markedly reduced Erk1/2 inhibition and p38 MAPK phosphorylation induced by DNCB and thimerosal, suggesting a direct activation of p38 MAPK via an oxidative stress and a regulation of MAPK signalling pathways depending on chemicals. Because of a high sensitivity of kinase activity measurements, these procedures will be suitable for weak or moderate sensitizer screening.

J Toxicol. 2012;2012:373678. doi: 10.1155/2012/373678. Epub 2012 Jun 28.

## Thimerosal-Derived Ethylmercury Is a Mitochondrial Toxin in Human Astrocytes: Possible Role of Fenton Chemistry in the Oxidation and Breakage of mtDNA.

[Sharpe MA](#)<sup>1</sup>, [Livingston AD](#), [Baskin DS](#).

Thimerosal generates ethylmercury in aqueous solution and is widely used as preservative. We have investigated the toxicology of Thimerosal in normal human astrocytes, paying particular attention to mitochondrial function and the generation of specific oxidants. We find that ethylmercury not only inhibits mitochondrial respiration leading to a drop in the steady state membrane potential, but also concurrent with these phenomena increases the formation of superoxide, hydrogen peroxide, and Fenton/Haber-Weiss generated hydroxyl radical. These oxidants increase the levels of cellular aldehyde/ketones. Additionally, we find a five-fold increase in the levels of oxidant damaged mitochondrial DNA bases and increases in the levels of mtDNA nicks and blunt-ended breaks. Highly damaged mitochondria are characterized by having very low membrane potentials, increased superoxide/hydrogen peroxide production, and extensively damaged mtDNA and proteins. These mitochondria appear to have undergone a permeability transition, an observation supported by the five-fold increase in Caspase-3 activity observed after Thimerosal treatment.

## Mitochondrial mediated thimerosal-induced apoptosis in a human neuroblastoma celline (SK-N-SH)

*Humphrey ML, Cole MP, Pendergrass JC, Kiningham KK. Neurotoxicology. 2005 Jun;26(3):407-16.*

Environmental exposure to mercurials continues to be a public health issue due to their deleterious effects on immune, renal and neurological function. Recently the safety of thimerosal, an ethyl mercury-containing preservative used in vaccines, has been questioned due to exposure of infants during immunization. Mercurials have been reported to cause apoptosis in cultured neurons; however, the signaling pathways resulting in cell death have not been well characterized. Therefore, the objective of this study was to identify the mode of cell death in an in vitro model of thimerosal-induced neurotoxicity, and more specifically, to elucidate signaling pathways which might serve as pharmacological targets. Within 2 h of thimerosal exposure (5  $\mu$ M) to the human neuroblastoma cell line, SK-N-SH, morphological changes, including membrane alterations and cell shrinkage, were observed. Cell viability, assessed by measurement of lactate dehydrogenase (LDH) activity in the medium, as well as the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, showed a time- and concentration-dependent decrease in cell survival upon thimerosal exposure. In cells treated for 24 h with thimerosal, fluorescence microscopy indicated cells undergoing both apoptosis and oncosis/necrosis. To identify the apoptotic pathway associated with thimerosal-mediated cell death, we first evaluated the mitochondrial cascade, as both inorganic and organic mercurials have been reported to accumulate in the organelle. Cytochrome *c* was shown to leak from the mitochondria, followed by caspase 9 cleavage within 8 h of treatment. In addition, poly(ADP-ribose) polymerase (PARP) was cleaved to form a 85 kDa fragment following maximal caspase 3 activation at 24 h.

Taken together these findings suggest deleterious effects on the cytoarchitecture by thimerosal and initiation of mitochondrial-mediated apoptosis

### Mitochondrial dysfunction, impaired oxidative-reduction activity, degeneration, and death in human neuronal and fetal cells induced by low-level exposure to thimerosal and other metal compounds

*D.A. Geier et al. Toxicological & Environmental Chemistry. 2009, 1–15, iFirst*

Thimerosal (ethylmercurithiosalicylic acid), an ethylmercury (EtHg)-releasing compound (49.55% mercury (Hg)), was used in a range of medical products for more than 70 years. Of particular recent concern, routine administering of Thimerosal-containing biologics/childhood vaccines have become significant sources of Hg exposure for some fetuses/infants. This study was undertaken to investigate cellular damage among in vitro human neuronal (SH-SY-5Y neuroblastoma and 1321N1 astrocytoma) and fetal (nontransformed) model systems using cell vitality assays and microscope-based digital image capture techniques to assess potential damage induced by Thimerosal and other metal compounds (aluminum (Al) sulfate, lead (Pb)(II) acetate, methylmercury (MeHg) hydroxide, and mercury (Hg)(II) chloride) where the cation was reported to exert adverse effects on developing cells. Thimerosal-associated cellular damage was also evaluated for similarity to pathophysiological findings observed in patients diagnosed with autistic disorders (ADs). Thimerosal-induced cellular damage as evidenced by concentration- and time-dependent mitochondrial damage, reduced oxidative-reduction activity, cellular degeneration, and cell death in the in vitro human neuronal and fetal model systems studied. Thimerosal at low nanomolar (nM) concentrations induced significant cellular toxicity in human neuronal and fetal cells. Thimerosal-induced cytotoxicity is similar to that observed in AD pathophysiologic studies. Thimerosal was found to be significantly more toxic than the other metal compounds examined. Future studies need to be conducted to evaluate additional mechanisms underlying Thimerosal-induced cellular damage and assess potential co-exposures to other compounds that may increase or decrease Thimerosal-mediated toxicity.

### Sensitization effect of thimerosal is mediated in vitro via reactive oxygen species and calcium signaling.

*Migdal C, Foggia L, Tailhardat M, Courtellemont P, Haftek M, Serres M. Toxicology. 2010 Jul-Aug;274(1-3):1-9. Epub 2010 May 10.*

Thimerosal, a mercury derivative composed of ethyl mercury chloride (EtHgCl) and thiosalicylic acid (TSA), is widely used as a preservative in vaccines and cosmetic products and causes cutaneous reactions. Since dendritic cells (DCs) play an essential role in the immune response, the sensitization potency of chemicals was studied in vitro using U937, a human promyelomonocytic cell line that is used as a surrogate of monocytic differentiation and

activation. Currently, this cell line is under ECVAM (European Center for the Validation of Alternative Methods) validation as an alternative method for discriminating chemicals. Thimerosal and mercury derivatives induced in U937 an overexpression of CD86 and interleukin (IL)-8 secretion similarly to 1-chloro-2,4-dinitrobenzene (DNCB), a sensitizer used as a positive control for DC activation. Non-sensitizers, dichloronitrobenzene (DCNB), TSA and sodium dodecyl sulfate (SDS), an irritant, had no effect. U937 activation was prevented by cell pretreatment with N-acetyl-L-cysteine (NAC) but not with thiol-independent antioxidants except vitamin E which affected CD86 expression by preventing lipid peroxidation of cell membranes. Thimerosal, EtHgCl and DNCB induced glutathione (GSH) depletion and reactive oxygen species (ROS) within 15 min; another peak was detected after 2h for mercury compounds only. MitoSOX, a specific mitochondrial fluorescent probe, confirmed that ROS were essentially produced by mitochondria in correlation with its membrane depolarization. Changes in mitochondrial membrane permeability induced by mercury were reversed by NAC but not by thiol-independent antioxidants. Thimerosal and EtHgCl also induced a calcium (Ca<sup>2+</sup>) influx with a peak at 3h, suggesting that Ca<sup>2+</sup> influx is a secondary event following ROS induction as Ca<sup>2+</sup> influx was suppressed after pretreatment with NAC but not with thiol-independent antioxidants. Ca<sup>2+</sup> influx was also suppressed when culture medium was deprived of Ca<sup>2+</sup> confirming the specificity of the measure. In conclusion, these data suggest that thimerosal induced U937 activation via oxidative stress from mitochondrial stores and mitochondrial membrane depolarization with a primordial effect of thiol groups. A cross-talk between ROS and Ca<sup>2+</sup> influx was demonstrated.

## [Evaluation of cytotoxicity attributed to thimerosal on murine and human kidney cells.](#)

*Park EK, Mak SK, Kültz D, Hammock BD. J Toxicol Environ Health A. 2007 Dec;70(24):2092-5.*

Renal inner medullary collecting duct cells (mIMCD3) and human embryonic kidney cells (HEK293) were used for cytoscreening of thimerosal and mercury chloride (HgCl<sub>2</sub>). Thimerosal and HgCl<sub>2</sub> acted in a concentration-dependent manner. In mIMCD3 cells the 24-h LC<sub>50</sub> values for thimerosal, thiosalicylic acid, 2,2-dithiosalicylic acid, and 2-sulfobenzoic acid were 2.9, 2200, >1000, and >10,000 microM, respectively. The 24-h LC<sub>50</sub> value for HgCl<sub>2</sub> in mIMCD3 cells was 40 microM. In HEK293 cells, the 24-h LC<sub>50</sub> value for thimerosal was 9.5 microM. These data demonstrate that the higher cytotoxicity produced by thimerosal on renal cells with respect to similar compounds without Hg may be related to this metal content. The present study also establishes mIMCD3 cells as a valuable model for evaluation of cytotoxicity of nephrotoxic compounds.

## [The relative toxicity of compounds used as preservatives in vaccines and biologics.](#)

*Geier DA, Jordan SK, Geier MR. Med Sci Monit. 2010 Apr 28;16(5):SR21-7.*

**BACKGROUND:** In vaccines/biologics, preservatives are used to prevent microbial growth.

**MATERIAL/METHODS:** The present study examined: (1) the comparative toxicities of commonly used preservatives in US licensed vaccines to human neurons; and (2) the relative toxicity index of these compounds to human neurons in comparison to bacterial cells.

**RESULTS:** Using human neuroblastoma cells, the relative cytotoxicity of the levels of the compounds commonly used as preservative in US licensed vaccines was found to be phenol < 2-phenoxyethanol < benzethonium chloride < Thimerosal. The observed relative toxicity indices (human neuroblastoma cells/bacterial cells) were 2-phenoxyethanol (4.6-fold) < phenol (12.2-fold) < Thimerosal (>330-fold). In addition, for the compounds tested, except for 2-phenoxyethanol, the concentrations necessary to induce significant killing of bacterial cells were significantly higher than those routinely present in US licensed vaccine/biological preparations.

**CONCLUSIONS:** None of the compounds commonly used as preservatives in US licensed vaccine/biological preparations can be considered an ideal preservative, and their ability to fully comply with the requirements of the US Code of Federal Regulations (CFR) for preservatives is in doubt. Future formulations of US licensed vaccines/biologics should be produced in aseptic manufacturing plants as single dose preparations, eliminating the need for preservatives and an unnecessary risk to patients.

## [Low molecular weight thiols reduce thimerosal neurotoxicity in vitro: Modulation by proteins.](#)

*Zieminska E, Toczylowska B, Stafiej A, Lazarewicz JW. Toxicology. 2010 Aug 7. [Epub ahead of print]*

Thimerosal (TH), an ethylmercury complex of thiosalicylic acid has been used as preservative in vaccines. In vitro neurotoxicity of TH at high nM concentrations has been reported. Although a number of toxicological experiments demonstrated high affinity of mercury to thiol groups of the extracellular amino acids and proteins that may decrease concentration of free TH in the organism, less is known about the role of interactions between proteins and amino acids in protection against TH neurotoxicity. In the present study we examined whether the presence of serum proteins and of l-cysteine (Cys), d,l-homocysteine (Hcy), N-acetyl cysteine (NAC), l-methionine (Met) and glutathione (GSH) in the incubation medium affects the TH-induced changes in the viability, the intracellular levels of calcium and zinc and mitochondrial membrane potential in primary cultures of rat cerebellar granule cells. The cells were exposed to 500nM TH for 48h or to 15-25µM TH for 10min. Our results demonstrated a decrease in the cells viability evoked by TH, which could be prevented partially by serum proteins, albumin or in a dose-dependent manner by 60, 120 or 600µM Cys, Hcy, NAC and GSH, but not by Met. This neuroprotection was less pronounced in the presence of proteins. Incubation of neurons with TH also induced the rise in the intracellular calcium and zinc concentration and decrease in mitochondrial membrane potential, and these effects were abolished by all the sulfur containing compounds studied and administered at 600µM concentration, except Met. The loss of the ethylmercury moiety from TH as a result of interaction with thiols studied was monitored by (1)H NMR spectroscopy. This extracellular process may be responsible for the neuroprotection seen in the cerebellar cell cultures, but also provides a molecular pathway for redistribution of TH-derived toxic ethylmercury in the organism. In conclusion, these results confirmed that

proteins and sulfur-containing amino acids applied separately reduce TH neurotoxicity, while their combination modulates in more complex way neuronal survival in the presence of TH.

## [Mercury induces an unopposed inflammatory response in human peripheral blood mononuclear cells in vitro.](#)

*Gardner RM, Nyland JF, Evans SL, Wang SB, Doyle KM, Crainiceanu CM, Silbergeld EK. Environ Health Perspect. 2009 Dec;117(12):1932-8. Epub 2009 Aug 19.*

**BACKGROUND:** The human immune response to mercury is not well characterized despite the body of evidence that suggests that Hg can modulate immune responses, including the induction of autoimmune disease in some mouse models. Dysregulation of cytokine signaling appears to play an important role in the etiology of Hg-induced autoimmunity in animal models.

**OBJECTIVES:** In this study, we systematically investigated the human immune response to Hg in vitro in terms of cytokine release. **METHODS:** Human peripheral blood mononuclear cells (PBMCs) were isolated from 20 volunteers who donated blood six separate times. PBMCs were cultured with lipopolysaccharide and concentrations of mercuric chloride (HgCl<sub>2</sub>) up to 200 nM. Seven cytokines representing important pathways in physiologic and pathologic immune responses were measured in supernatants. We used multilevel models to account for the intrinsic clustering in the cytokine data due to experimental design. **RESULTS:** We found a consistent increase in the release of the proinflammatory cytokines interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha, and concurrent decrease in release of the antiinflammatory cytokines interleukin 1-receptor antagonist (IL-1Ra) and IL-10 in human PBMCs treated with subcytotoxic concentrations of HgCl<sub>2</sub>. IL-4, IL-17, and interferon-gamma increased in a concentration-response manner. These results were replicated in a second, independently recruited population of 20 different volunteers. **CONCLUSIONS:** Low concentrations of HgCl<sub>2</sub> affect immune function in human cells by dysregulation of cytokine signaling pathways, with the potential to influence diverse health outcomes such as susceptibility to infectious disease or risk of autoimmunity.

WMP Note: Given these results, the equivalent study should be done using thimerosal.

## [Responsiveness of human monocyte-derived dendritic cells to thimerosal and mercury derivatives.](#)

*Migdal C, Tailhardat M, Courtellemont P, Haftek M, Serres M. Toxicol Appl Pharmacol. 2010 Jul;246(1-2):66-73. Epub 2010 Apr 22.*

Several cases of skin sensitization have been reported following the application of thimerosal, which is composed of ethyl mercury and thiosalicylic acid (TSA). However, few in vitro studies have been carried out on human dendritic cells (DCs) which play an essential role in the initiation of allergic contact dermatitis. The aim of the present study was to identify the effect of thimerosal and other mercury compounds on human DCs. To address this purpose, DCs derived

from monocytes (mono-DCs) were used. Data show that thimerosal and mercury derivatives induced DC activation, as monitored by CD86 and HLA-DR overexpression associated with the secretion of tumor necrosis factor alpha and interleukin 8, similarly to lipopolysaccharide and the sensitizers, 1-chloro-2,4-dinitrobenzene (DNCB) and nickel sulfate, which were used as positive controls. In contrast, TSA, the non-mercury part of thimerosal, as well as dichloronitrobenzene, a DNCB negative control, and the irritant, sodium dodecyl sulfate, had no effect. Moreover, oxidative stress, monitored by ROS induction and depolarization of the mitochondrial membrane potential, was induced by thimerosal and mercury compounds, as well as DNCB, in comparison with hydrogen peroxide, used as a positive control. The role of thiol oxidation in the initiation of mono-DC activation was confirmed by a pre-treatment with N-acetyl-L-cysteine which strongly decreased chemical-induced CD86 overexpression. These data are in agreement with several clinical observations of the high relevance of thimerosal in patch-test reactions and prove that human mono-DCs are useful in vitro tools for determining the allergenic potency of chemicals.