

**Summary of Science on Thimerosal Effects at Vaccine-Relevant Doses
Submission to the Colorado Senate
Heath & Human Services Committee
for its deliberation of SB 06-099
February 22, 2006**

Thimerosal has powerful effects on cells of the nervous and immune systems in mammals including humans. Its effect may vary depending on the dose, the genetics of the individual, and the timing of exposure. Attached is a brief summary for the Committee of the recently published research on thimerosal at low doses close to or equal to that found in vaccines or at concentrations that are likely to result from vaccine administration. This research strongly suggests that thimerosal from vaccines given to infants or pregnant women has the potential to cause harmful effects. More research is needed, but in the interest of precaution, removal of mercury from vaccines given to vulnerable populations is warranted. We support actions that lead to such removal, especially since sufficient supplies of mercury free vaccines are readily available.

Signed

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RESEARCH SUMMARY

Note: the mercury dose from vaccines produces acute ethylmercury blood levels in the nanomolar range. The half life is 5-7 days, meaning that half the injected dose of mercury leaves the blood in that time period, on average. There is considerable individual variation. Any background mercury exposures from non-vaccine sources would increase the blood mercury levels.

1. **Baskin (2003)** – thimerosal disrupts cell membranes, damages DNA, and alters cell shape at concentrations only 4 times those expected from vaccines. Greater effects were seen as the length of time of exposure grew, suggesting that under real conditions the concentration needed for the observed alterations would be much lower. This has been shown in subsequent research, that exposure of cells to nanomolar levels of thimerosal after 24 hours results in cell alterations.
2. **Burbacher (2005)**: infant monkeys dosed with vaccine-level thimerosal were compared with infant monkeys dosed with equal levels of methylmercury. The thimerosal dose resulted in lower blood levels but more than twice the inorganic, or long term, mercury levels in the brain, relative to the methylmercury. The study showed the potential for significant brain accumulation from thimerosal and demonstrated that exposure/safety assessments for methylmercury may not apply to thimerosal.
3. **Havarinasab & Hultman (2005)**: thimerosal given to mice alters immune function more than equal doses of methylmercury.
4. **Hornig (2005)**: dosing of autoimmune-prone infant mice with thimerosal-containing vaccines, at the dose given to humans adjusted for mouse weight, resulted in a number of observable effects including growth delay, reduced movement, exaggerated responses, and brain alterations such as increased neuron density and changes in receptors and transporters.
5. **Humphrey & Kinningham (2005)**: after only short (2 hour) exposures, thimerosal at micromolar concentrations caused neuronal membrane damage and alterations leading to cell death.
6. **James (2005)**: the viability of neuronal cell lines was decreased after just 3 hour exposure to 2.5 micromolar concentrations of thimerosal.
7. **Makani & Yel (2002)** – thimerosal at micromolar amounts causes cell death (apoptosis) in immune cells (T cells).
8. **Mutkus & Aschner (2005)** – thimerosal alters glutamate transporter function at low micromolar concentrations. Glutamate is a neurotransmitter and is necessary for proper brain functioning.
9. **Parran (2005)**- thimerosal causes DNA fragmentation of neuronal cells and disrupts neuronal growth factor signaling at micromolar and even nanomolar concentrations.
10. **Ueha-Ishibashi (2004)**: thimerosal at low concentrations is as toxic to rat neurons as methylmercury. The FDA and EPA use methylmercury as their toxicity standard, so demonstration of equivalence shows the potential of thimerosal to cause the same harm as methylmercury, for which more research exists.
11. **Waly & Deth (2004)**: thimerosal inhibits critical DNA methylation and attentional pathways at nanomolar concentrations, leading to alterations in brain function.
12. **Westphal (2003)** – thimerosal at nanomolar concentrations causes DNA damage in immune cells (lymphocytes) leading to cell death.

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6. James SJ, Slikker W 3rd, Melnyk S, New E, Pogribna M, Jernigan S. Thimerosal neurotoxicity is associated with glutathione depletion: protection with glutathione precursors. *Neurotoxicology*. 2005 Jan;26(1):1-8.
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11. 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. Activation of methionine synthase by insulin-like growth factor-1 and dopamine: a target for neurodevelopmental toxins and thimerosal. *Molecular Psychiatry*. 2004 Apr;9(4):358-70.
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RESEARCH ABSTRACTS

Baskin DS, Ngo H, Didenko VV. Thimerosal induces DNA breaks, caspase-3 activation, membrane damage, and cell death in cultured human neurons and fibroblasts. *Toxicological Sciences*. 2003 Aug;74(2):361-8. Epub 2003 May 28.

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-microM 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 microM** based on the manual detection of the fluorescent attached cells and at a 1-microM 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 microM 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.

Burbacher TM, Shen DD, Liberato N, Grant KS, Cernichiari E, Clarkson T. Comparison of blood and brain mercury levels in infant monkeys exposed to methylmercury or vaccines containing thimerosal. *Environmental Health Perspectives*. 2005 Aug;113(8):1015-21.

Thimerosal is a preservative that has been used in manufacturing vaccines since the 1930s. Reports have indicated that infants can receive ethylmercury (in the form of thimerosal) at or above the U.S. Environmental Protection Agency guidelines for methylmercury exposure, depending on the exact vaccinations, schedule, and size of the infant. In this study we compared the systemic disposition and brain distribution of total and inorganic mercury in infant monkeys after thimerosal exposure with those exposed to MeHg. Monkeys were exposed to MeHg (via oral gavage) or vaccines containing thimerosal (via intramuscular injection) at birth and 1, 2, and 3 weeks of age. Total blood Hg levels were determined 2, 4, and 7 days after each exposure. Total and inorganic brain Hg levels were assessed 2, 4, 7, or 28 days after the last exposure. The initial and

terminal half-life of Hg in blood after thimerosal exposure was 2.1 and 8.6 days, respectively, which are significantly shorter than the elimination half-life of Hg after MeHg exposure at 21.5 days. Brain concentrations of total Hg were significantly lower by approximately 3-fold for the thimerosal-exposed monkeys when compared with the MeHg infants, whereas the average brain-to-blood concentration ratio was slightly higher for the thimerosal-exposed monkeys (3.5 +/- 0.5 vs. 2.5 +/- 0.3). A higher percentage of the total Hg in the brain was in the form of inorganic Hg for the thimerosal-exposed monkeys (34% vs. 7%). The results indicate that MeHg is not a suitable reference for risk assessment from exposure to thimerosal-derived Hg. Knowledge of the toxicokinetics and developmental toxicity of thimerosal is needed to afford a meaningful assessment of the developmental effects of thimerosal-containing vaccines.

Havarinasab S, Hultman P. Organic mercury compounds and autoimmunity. *Autoimmun Rev.* 2005 Jun;4(5):270-5. Epub 2005 Jan 5.

Based on in vitro studies and short-term in vivo studies, all mercurials were for a long time considered as prototypic immunosuppressive substances. Recent studies have confirmed that organic mercurials such as methyl mercury (MeHg) and ethyl mercury (EtHg) are much more potent immunosuppressors than inorganic mercury (Hg). However, Hg interacts with the immune system in the presence of a susceptible genotype to cause immunostimulation, antinucleolar antibodies targeting fibrillarin, and systemic immune-complex (IC) deposits, a syndrome called Hg-induced autoimmunity (HgIA). Recent studies in mice with a susceptible genotype has revealed that the immunosuppressive effect of MeHg and EtHg will within 1-3 weeks be superseded by immunostimulation causing an HgIA-like syndrome. At equimolar doses of Hg, MeHg has the weakest immunostimulating, autoimmunogen, and IC-inducing effect, while the effect of thimerosal is similar to that of inorganic mercury. The immunosuppression is caused by the organic mercurials per se. Since they undergo rapid transformation to inorganic Hg, studies are being undertaken to delineate the importance of the organic substances per se and the newly formed inorganic Hg for induction of autoimmunity.

Hornig M, Chian D, Lipkin WI. Neurotoxic effects of postnatal thimerosal are mouse strain dependent. *Molecular Psychiatry.* 2004 Sep;9(9):833-45.

The developing brain is uniquely susceptible to the neurotoxic hazard posed by mercurials. Host differences in maturation, metabolism, nutrition, sex, and autoimmunity influence outcomes. How population-based variability affects the safety of the ethylmercury-containing vaccine preservative, thimerosal, is unknown. Reported increases in the prevalence of autism, a highly heritable neuropsychiatric condition, are intensifying public focus on environmental exposures such as thimerosal. Immune profiles and family history in autism are frequently consistent with autoimmunity. We hypothesized that autoimmune propensity influences outcomes in mice following thimerosal challenges that mimic routine childhood immunizations. Autoimmune disease-sensitive SJL/J mice showed growth delay; reduced locomotion; exaggerated

response to novelty; and densely packed, hyperchromic hippocampal neurons with altered glutamate receptors and transporters. Strains resistant to autoimmunity, C57BL/6J and BALB/cJ, were not susceptible. These findings implicate genetic influences and provide a model for investigating thimerosal-related neurotoxicity.

Humphrey ML, Cole MP, Pendergrass JC, Kinningham KK. Mitochondrial mediated thimerosal-induced apoptosis in a human neuroblastoma cell line (SK-N-SH). *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 microM) 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.

James SJ, Slikker W 3rd, Melnyk S, New E, Pogribna M, Jernigan S. Thimerosal neurotoxicity is associated with glutathione depletion: protection with glutathione precursors. *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 microM 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 microM 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.

Makani S, Gollapudi S, Yel L, Chiplunkar S, Gupta S. Biochemical and molecular basis of thimerosal-induced apoptosis in T cells: a major role of mitochondrial pathway. *Genes Immun.* 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.**

Mutkus L, Aschner JL, Syversen T, Shanker G, Sonnewald U, Aschner M. In vitro uptake of glutamate in GLAST- and GLT-1-transfected mutant CHO-K1 cells is inhibited by the ethylmercury-containing preservative thimerosal. *Biological Trace Element Research.* 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⁺-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 [3H]-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.**

Parran DK, Barker A, Ehrich M. Effects of thimerosal on NGF signal transduction and cell death in neuroblastoma cells. *Toxicological Sciences*. 2005 Jul;86(1):132-40. Epub 2005 Apr 20.

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 microM), 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 microM (apoptosis) to decrease at concentrations >1 microM (necrosis). These data demonstrate that thimerosal could alter NGF-induced signaling in neurotrophin-treated cells at concentrations lower than those responsible for cell death.**

Ueha-Ishibashi T, Oyama Y, Nakao H, Umabayashi C, Nishizaki Y, Tatsuishi T, Iwase K, Murao K, Seo H. Effect of thimerosal, a preservative in vaccines, on intracellular Ca^{2+} concentration of rat cerebellar neurons. *Toxicology*. 2004 Jan 15;195(1):77-84.

The effect of thimerosal, an organomercurial preservative in vaccines, on cerebellar neurons dissociated from 2-week-old rats was compared with those of methylmercury using a flow cytometer with appropriate fluorescent dyes. Thimerosal and methylmercury at concentrations ranging from 0.3 to 10 μM increased the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) in a concentration-dependent manner. The potency of 10 μM thimerosal to increase the $[\text{Ca}^{2+}]_i$ was less than that of 10 μM methylmercury. Their effects on the $[\text{Ca}^{2+}]_i$ were greatly attenuated, but not completely suppressed, under external Ca^{2+} -free condition, suggesting a possibility that both agents increase membrane Ca^{2+} permeability and release Ca^{2+} from intracellular calcium stores. The effect of 10 μM thimerosal was not affected by simultaneous application of 30 μM L-cysteine whereas that of 10 μM methylmercury was significantly suppressed. The potency of thimerosal was similar to that of methylmercury in the presence of L-cysteine. Both agents at 1 μM or more similarly decreased the cellular content of glutathione in a concentration-dependent manner, suggesting an increase in oxidative stress. Results indicate that thimerosal exerts some cytotoxic actions on cerebellar granule neurons dissociated from 2-week-old rats and its potency is almost similar to that of methylmercury.

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. Activation of methionine synthase by insulin-like growth factor-1 and dopamine: a target for neurodevelopmental toxins and thimerosal. *Molecular 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.

Götz A. Westphal, Soha Asgari, Thomas G. Schulz, Jürgen Bünger, Michael Müller, Ernst Hallier Thimerosal induces micronuclei in the cytochalasin B block micronucleus test with human lymphocytes. *Archives of Toxicology*. 2003 Jan; 77(1):50 – 55.

Thimerosal is a widely used preservative in health care products, especially in vaccines. Due to possible adverse health effects, investigations on its metabolism and toxicity are urgently needed. An in vivo study on chronic toxicity of thimerosal in rats was inconclusive and reports on genotoxic effects in various in vitro systems were contradictory. Therefore, we reinvestigated thimerosal in the cytochalasin B block micronucleus test. Glutathione S-transferases were proposed to be involved in the detoxification of thimerosal or its decomposition products. Since the outcome of genotoxicity studies can be dependent on the metabolic competence of the cells used, we were additionally interested whether polymorphisms of glutathione S-transferases (GSTM1, GSTT1, or GSTP1) may influence the results of the micronucleus test with primary human lymphocytes. Blood samples of six healthy donors of different glutathione S-transferase genotypes were included in the study. At least two independent experiments were performed for each blood donor. Significant induction of micronuclei was seen at concentrations 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. These data raise some concern on the widespread use of thimerosal.