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Thimerosal Induces DNA Breaks, Caspase-3 Activation, Membrane Damage, and Cell Death in Cultured Human Neurons and Fibroblasts

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Abstract

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- μ M concentrations of thimerosal for 45 min to 24 h. A 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) dye exclusion test was used to identify non-viable 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 μ M based on the manual detection of the fluorescent attached cells and at a 1- μ M 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 μ M 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.

Keywords

thimerosal; active caspase-3; apoptosis; toxicity; neurons; fibroblasts; DNA breaks; membrane damage; DAPI

Thimerosal (sodium ethylmercury-thiosalicylate) is an antibacterial and antifungal mercurial compound used as a preservative in biological products and vaccines, in concentrations ranging from 0.003 to 0.01% (30–100 μ g/ml) (Ball *et al.*, 2001). **Thimerosal contains 49.6 % mercury by weight and releases ethylmercury as a metabolite. In the body, ethylmercury can be converted to inorganic mercury, which then preferentially accumulates in the kidneys and brain** (Blair *et al.*, 1975). Inorganic mercury is known to induce membrane and DNA damage (Ferrat *et al.*, 2002; Ben-Ozer *et al.*, 2000), and in cell culture conditions it was shown to be mutagenic and generate DNA breaks in concentrations below 500 nM (Schurz *et al.*, 2000). **Ethylmercury**

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can significantly increase the concentration of inorganic mercury in many organs (Magos *et al.*, 1985). After *in vivo* administration, ethylmercury passes through cellular membranes and concentrates in cells in vital organs, including the brain, where it releases inorganic mercury, raising its concentrations higher than equimolar doses of its close and highly toxic relative methylmercury (Magos *et al.*, 1985).

However, little is known about acute reactions of various types of human cells following short-time exposure to thimerosal in micro- and nanomolar concentrations.

In this paper we used a convenient and easily reproducible combination of fluorescent techniques analyzing various markers of DNA and membrane damage, and investigated the toxicity of micromolar and nanomolar concentrations of thimerosal (125 nM–250 μ M) occurring in the first 24 h of exposure in cultures of human cortical neuronal cells and in human fibroblasts.

We found that thimerosal in micromolar concentrations rapidly decreased cellular viability. Within several h after thimerosal administration, cells lost their capability to exclude the fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and developed multiple DNA breaks accompanied by caspase-3 activation and apoptotic morphology. Neuronal cell cultures demonstrated a higher sensitivity to thimerosal compared with fibroblasts.

MATERIALS AND METHODS

Cell cultures

HCN-1A Human cerebral cortical neurons (CRL-10442) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to ATCC recommendations. The line was derived from cortical tissue removed from a patient undergoing hemispherectomy for intractable seizures. As recommended by ATCC, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-glutamine, modified to contain 4.5g/l glucose and 1.5g/l sodium bicarbonate, supplemented with 10% fetal bovine serum, and the pH adjusted to 7.35 prior to filtration.

Normal neonatal human foreskin HCA 2 fibroblasts (PD32) were obtained from the laboratory of Dr. Olivia Smith-Pereira, Ph.D. The cells were grown in DMEM supplemented with 10% fetal bovine serum medium, and the pH was adjusted to 7.4 prior to filtration. For the experiments, all cells were subcultured in 24-well cell culture plates (Fisher, Pittsburgh, PA). All experiments were reproduced in triplicates. Each of the parallel series yielded identical results.

Thimerosal

Thimerosal (minimum 97% HPLC), SigmaUltra (Sigma, St. Louis, MO) was added to cell cultures in 30 μ l of double-distilled water to final concentrations of 250 μ M, 50 μ M, 10 μ M, 2 μ M, 1 μ M, 500 nM, 250 nM, and 125 nM. Concentrations of 1 μ M–125 nM were used with neuronal cells only. Control cell cultures received 30 μ l of water without thimerosal.

Dye exclusion test using DAPI

DAPI is a nonintercalating DNA-specific dye with an emission maximum in the blue spectrum (Shapiro, 1985). It is widely used for counterstaining cellular nuclei in fixed sections and has been demonstrated to be useful for the detection of nonviable cells with compromised membranes in live cell cultures (Boutonnat *et al.*, 1999; McCarthy and Hale, 1988).

The DAPI exclusion test was performed as described (Boutonnat *et al.*, 1999). Briefly, cells were incubated with DAPI (Sigma, St. Louis, MO) diluted in a cell culture medium at a final concentration of 100 ng/ml for 30 min at 20°C (Boutonnat *et al.*, 1999). A fluorescent signal was monitored and representative images were taken at 45 min and 2, 4, 6, and 24 h after the addition of thimerosal. The DAPI incubation started 30 min before each observation was made (at 15 min, 90 min, etc). Images were acquired using an Olympus IX-70 fluorescent microscope equipped with a MicroMax digital camera system (Princeton Instruments, Inc., Trenton, NJ) containing an RTE/CCD-1300-Y/HS array cooled by a Peltier device. Image acquisition was performed using the *MetaMorph* 4.1 program (Advanced Scientific, Inc., Meraux, LA). The micrographs were taken at central parts of the wells, where cellular density was most uniform.

Terminal transferase-based nick-end labeling (TUNEL)

Cells were fixed in ice cold methanol, and TUNEL staining for detection of DNA breaks was performed using the ApoTaq Fluorescein and ApoTaq Rhodamine kits for indirect immunofluorescence (Serologicals, Gaithersburg, MD), employing the standard technique recommended by the manufacturer. Following washing, the cells were counterstained with the DNA binding dye DAPI (1 µg/ml) for visualization of all cellular nuclei and were mounted in Vectashield (Vector Laboratories, Burlingame, CA) for observation by fluorescence microscopy.

Caspase-3 detection

Detection of active caspase-3 in live cell cultures was performed using an APO LOGIX™ carboxyfluorescein (FAM) caspase detection kit (Cell Technology, Minneapolis, MN). The kit detects active caspases in living cells through the use of a FAM-labeled DEVD fluoromethyl ketone (FMK) caspase inhibitor, which irreversibly binds to active caspase-3 (Amstad *et al.*, 2000; Bedner *et al.*, 2000; Smolewski *et al.*, 2001). The inhibitor is cell permeable and noncytotoxic. With lesser affinity, FAM-DEVD-FMK binds to the other caspases participating in apoptosis: caspase-8 > caspase-7 > caspase-10 > caspase-6 in the order of decreasing binding affinity (Carcia-Calvo *et al.*, 1998).

The kit was used as recommended by the manufacturer. Briefly, 10 µl of 30X Working Dilution FAM-Peptide-FMK was added to 300 µl of cell culture medium/per well, directly in 24-well cell culture plates after 5 h or 23 h of incubation with thimerosal. Cells were incubated for 1 h at 37°C under 5% CO₂, protected from light. Then the medium was carefully removed, and the cells were washed twice with 2 ml/per well of 1X Working Dilution Wash Buffer. The fluorescent signal was observed under an Olympus IX-70 fluorescent microscope equipped with a MicroMax digital camera system (Princeton Instruments, Inc.) containing an RTE/CCD-1300-Y/HS array cooled by a Peltier device. Caspase-positive cells appeared fluorescing green. Representative images were taken at 6 h and 24 h after the addition of thimerosal. Image acquisition was performed using the *MetaMorph* 4.1 program (Advanced Scientific, Inc.). Positive controls included cultures of cortical neurons treated with 0.5 µM staurosporin to induce caspase-3 activation. In several series of experiments, we added DAPI to cell cultures to the concentration of 100 ng/ml for 30 min immediately after 1 h of incubation with the FAM-Peptide-FMK solution. This made the co-localization of active caspase-3 and DAPI signals possible.

Fluorescence measurements using a microplate reader

In a separate set of experiments, we measured both active caspase-3 and DAPI signals in co-localization experiments using a GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence (Tecan Inc., Research Triangle Park, NC). Neuronal cells were incubated with 1–250 µM concentrations of thimerosal for 6 h and processed as described for simultaneous DAPI and active caspase-3 detection. Both FITC and DAPI fluorescence were

measured directly in 24-well plates using a Chroma Technology bandpass filter set: FITC excitation D490/40, emission 520/10; DAPI excitation D360/40, emission 460/20. The reactions were repeated twice and yielded the same dose-dependent increase in thimerosal toxicity. Background fluorescence was subtracted from the experimental series, and the results were represented as graphs of average values using Microsoft Excel.

RESULTS

Thimerosal-Induced Changes in Membrane Permeability and Cell Viability

Changes in cell viability rapidly occurred after administration of thimerosal in all cell cultures and were detected by the loss of ability to exclude the fluorescent dye DAPI. DAPI is classified as a semipermeant dye, which requires a relatively short (30-min) exposure time of cell cultures to the dye prior to the signal observation in a DAPI exclusion test (Boutonnat *et al.*, 1999). Under these conditions, the dye has been shown to be useful for the detection of nonviable cells and can be utilized as a selective marker of membrane integrity. Indeed, it is a less toxic alternative to propidium iodide (PI) (Boutonnat *et al.*, 1999).

The results of the experiments show a dose- and time-dependent increase of membrane permeability to DAPI, first detected after 2 h of incubation with thimerosal and resulting in the penetration of the dye into the nuclei and DNA staining (Figs. 1 and 2). Figure 1 presents experiments performed on human cultured cortical neurons (HCN-1A) and shows that, after 2 h of incubation with thimerosal at a concentration of 250 μM , the DAPI penetrated through cellular membranes and stained the cellular nuclei. The inability to exclude the dye indicates the loss of cellular membrane integrity and cell death (Boutonnat *et al.*, 1999; McCarthy and Hale, 1988). After 4 h of incubation, thimerosal-induced membrane permeability and DNA staining were observed at a concentration of 10 μM . After 6 h of incubation with thimerosal, changes in membrane permeability were detected at concentrations as low as 2 μM , based on the appearance of DAPI-stained cells attached to the bottom of the wells. In control cell cultures, which were treated with DAPI alone, only sporadic dead cells were detected, and their numbers stayed the same 2, 4, and 6 h after the addition of DAPI (Fig. 1). There was no change in cell membrane permeability for DAPI for up to 24 h if no thimerosal was added.

We performed direct counts of DAPI-positive cells for the initial quantitative assessment of our results. We counted all DAPI-positive cells in two $\times 40$ fields of view for each of the thimerosal concentrations after 6 h of incubation. All counts were taken in central parts of the wells, where the cellular density was most uniform. The comparison with the average density of cells in these areas revealed that, at 2- μM thimerosal, 11% were DAPI-positive; at 10- μM thimerosal, 58% were DAPI-positive; at 50- μM thimerosal, 61% of the cells were DAPI-positive; and at 250- μM thimerosal, 100% of the neurons had compromised cellular membranes. In controls, less than 1% of the cells were DAPI positive, due to cell death naturally occurring in the cell cultures.

No changes in membrane permeability and DAPI staining were observed with thimerosal concentrations lower than 2 μM at times of incubation up to 24 h.

Since dying cells disattach from the bottom shortly after death and float in the media, they cannot be counted. This explains the similar numbers of DAPI-positive cells counted after 10- and 50- μM thimerosal treatments, and it could have some affect on the sensitivity of the lower limit of toxicity measurements. To address this issue and to take into consideration all DAPI-stained cells, we used a fluorescent microplate reader, which detects the fluorescence of both attached and floating dead cells (see Fig. 3). Using a GENios Plus Microplate Reader, we detected the lower limit of thimerosal toxicity for neuronal cells after 6 h of incubation to be at 1- μM concentration of thimerosal.

Experiments with cultured human fibroblasts produced similar results, although, when compared with neuronal cells, the fibroblasts demonstrated a slightly lower sensitivity to thimerosal toxicity by the DAPI exclusion test in terms of the number of DAPI-stained cells (Fig. 2).

Similar to neuronal cells, significant numbers of DAPI-stained nuclei were first observed after 2 h of incubation with thimerosal at 250 μM concentration in the fibroblast culture experiments (Fig. 2). After 4 h of incubation, nuclear staining was detected at 10- μM concentration of thimerosal. However, unlike the neuronal cells, the human fibroblasts did not show toxicity at 2- μM concentration of thimerosal after 6 h of incubation.

Detection of Thimerosal-Induced DNA Damage

We used TUNEL to detect DNA breaks generated in neurons and fibroblasts after 6 h of incubation with thimerosal. Following incubation, the cells were fixed, labeled by TUNEL, and counterstained by DAPI, which in these experiments was employed as a fluorescent DNA marker to visualize all cell nuclei in fixed cell cultures.

The results of these experiments are presented in Figure 4. The figure demonstrates that TUNEL-positive cells were detected in all cell cultures after 6 h of incubation, up to the concentration of 2 μM of thimerosal.

To determine if extending the time of incubation with thimerosal at concentrations below 2 μM would result in the generation of DNA breaks, we extended the time of incubation to 24 h in a separate series of experiments. After 24 h, a TUNEL signal was detected in neuronal cells at 1- μM concentration of thimerosal (versus 2 μM at 6 h) (not shown). Incubation of neuronal cells for 24 h with concentrations of thimerosal below 1 μM (125, 250, and 500 nM) did not produce a TUNEL signal.

Detection of Apoptotic Morphology in Thimerosal-Treated Cells

We performed a morphological evaluation of the fixed and fluorescently stained cell cultures after thimerosal treatment for the purpose of identifying apoptotic cells. To identify apoptotic morphology, the cells were fixed and then stained by DAPI. In this experiment, DAPI was employed not as a vital dye, as in our previous study, but rather as a fluorescent histological nuclear stain. Although DAPI is an important marker used in live cell cultures to selectively label nonviable cells (Boutonnat *et al.*, 1999; McCarthy and Hale, 1988), it is also frequently used in fixed cells to visualize nuclear morphology and apoptotic bodies. We used it for this purpose in these tests.

Apoptotic morphology was detected in thimerosal-treated cells. Figure 5 demonstrates that, after 6 h of incubation, both fibroblasts and neurons showed morphological signs of apoptosis, which included chromatin condensation on the nuclear membrane, the appearance of characteristic doughnut-shaped nuclei, different stages of apoptotic body formation, and freely positioned apoptotic bodies. After 6 h of incubation, apoptotic morphology was observed at concentrations as low as 2 μM of thimerosal (Fig. 5), whereas, at 24 h after incubation, similar apoptotic morphology was observed at concentrations as low as 1 μM .

To further confirm the apoptotic nature of cell death induced by thimerosal, we performed detection of active caspase-3, which is a sensitive and specific indicator of apoptosis.

Active Caspase-3 in Thimerosal-Treated Cells

Caspase-3 activation serves as a sensitive marker of apoptosis, developing through caspase-3-dependent mechanisms, which constitutes one of the most frequent apoptotic pathways. We

employed visualization of active caspase-3 directly in living cells through the use of a FAM-labeled peptide caspase inhibitor (FAM-Peptide-FMK) (see Materials and Methods).

We detected caspase-3-positive neuronal cells after 6 h of incubation with thimerosal at concentrations ranging from 250 to 2 μ M. The intensity of the signal was dose-dependent and much lower at the 2- μ M concentration, compared to higher concentrations, probably due to an earlier stage of caspase-3 activation (Fig. 6).

Assessment of 200 cells per well randomly, using the fluo-rescent microscope, revealed that active caspase-3 was expressed in 20% of the cells at 2- μ M thimerosal, 26% at 10- μ M thimerosal, 83% at 50- μ M thimerosal, and 97% of the neurons at 250- μ M thimerosal concentration. In the controls, less than 1% of the cells was caspase-3-positive, due to cell death naturally occurring in the cell cultures.

At 2- μ M thimerosal, the active caspase-3 signal was predominantly observed in the cytoplasm, which represents the early stage of its activation, whereas, at higher concentrations of thimerosal, the signal was detected in both the cytoplasm and the nuclei (Fig. 6). (Nuclear localization of active caspase-3 is characteristic for later stages of the apoptotic process.)

When we used a fluorescent microplate reader, which detects signals from the detached cells, we detected active caspase-3 activation at 1- μ M concentration of thimerosal after 6-h incubation, probably due to the added contribution from floating dead cells (Fig. 3).

When we extended the incubation time with thimerosal from 6 to 24 h, detectable numbers of attached cells with active caspase-3 were observed at 1- μ M concentration of thimerosal (Fig. 7). An active caspase-3 signal at 1- μ M concentration was cytoplasmic, demonstrating an earlier stage of caspase-3 activation. Interestingly, after 24 h of incubation, the neurons treated with 2- μ M thimerosal showed the migration of caspase-3 from the cytoplasm to the nuclei (Fig. 7). The majority of caspase-3-positive cells were also DAPI-positive, which indicates membrane damage occurring simultaneously with apoptotic response. However, at the higher 250- μ M concentration of thimerosal, a number of cells were only DAPI-positive without caspase-3 activation, demonstrating necrotic death (Fig. 7). We did not detect active caspase-3 at 24 h of incubation in untreated neurons, or in neuronal cultures treated with lower concentrations of thimerosal (500, 250, and 125 nM).

DISCUSSION

Our data indicate that thimerosal is toxic to human neurons and fibroblasts if applied in micromolar concentrations (1–250 μ M). An early sign of thimerosal toxicity is a change in cellular membrane permeability to the vital dye DAPI, which is associated with the loss of cell viability (Boutonnat *et al.*, 1999; McCarthy and Hale, 1988). This can be detected as early as 2 h after incubation.

DAPI proved to be useful for analyzing thimerosal toxicity, because it is a sensitive marker of membrane integrity. It is employed as a propidium iodide substitute in cell viability assays and labels nuclei of dying cells, which lack an intact plasma membrane (Boutonnat *et al.*, 1999; Castro-Hermida *et al.*, 2000; McCarthy and Hale, 1988; Robertson *et al.*, 1998). Dual staining experiments using propidium iodide and DAPI co-staining with FACS analysis demonstrated that DAPI stains only dead cells (McCarthy and Hale, 1988). Viable cells that are not stained by PI also exclude DAPI (McCarthy and Hale, 1988).

The nature of cell death labeled by DAPI in the case of thimerosal treatment deserves additional discussion. The DAPI exclusion method relies on the fact that this dye is largely impermeable to cells with an intact plasma membrane. However, when cell membrane integrity becomes

compromised, DAPI gains access to the nucleus, where it complexes with DNA and renders the nucleus highly fluorescent. Early compromised integrity of plasma membranes is a characteristic feature of necrotic cell death, whereas, in apoptosis, cellular membranes are compromised at later times. This is why intra-cellular staining by DAPI (and also by its more toxic substitute propidium iodide) is regularly interpreted as a sign of necrosis (Boutonnat *et al.*, 1999). However, in the case of thimerosal, the changes in membrane permeability coincided with the activation of apoptosis-specific caspase-3 (Fig. 3). In our opinion, this indicates a separate direct membrane damaging effect of thimerosal that developed simultaneously with apoptotic changes, such as caspase-3 activation.

In many cases, the importance of caspase-3 activation is related to its connection to specific and extensive apoptotic DNA cleavage (Porter and Janicke, 1999). This DNA fragmentation can be labeled by the TUNEL technique and is widely used for the visualization of apoptotic cells. A caspase-activated deoxyribonuclease (CAD, or DFF 40) is implicated as a direct executioner of the cleavage (Liu *et al.*, 1997; Mukae *et al.*, 1998). Most of the time, the enzyme is kept inactive by the binding of an inhibitor (ICAD, or DFF 45). Activation of the nuclease occurs when the inhibitor is cleaved by activated caspase-3 (Enari *et al.*, 1998; Sakahira *et al.*, 1998). However, the exact sequence of events in case of a human brain is likely different from this scheme. In human CNS neurons, other caspase-3-related pathways and possibly the other DNA cleaving enzymes are more important, and the role of the CAD-mediated mechanism is likely limited, because no expression of CAD mRNA was detected in human brain cells (Mukae *et al.*, 1998).

Similar to our results, high cellular toxicity of thimerosal in low micromolar concentrations was recently reported using another cell culture model (Makani *et al.*, 2002). The effects of different concentrations of thimerosal were examined in Jurkat cells. The cells were incubated with 5- to 0.5- μM concentrations of thimerosal for 24 h. Concentration-dependent apoptosis was detected and measured by TUNEL. Caspase-3 activation was also detected after 4 and 6 h of incubation with thimerosal. The study concluded that thimerosal induced caspase-3-dependent apoptosis in Jurkat cells. This apoptosis was associated with the depolarization of the mitochondrial membrane and release of cytochrome c. In this same study, a significantly enhanced generation of reactive oxygen species was also detected, as a result of incubation with thimerosal (Makani *et al.*, 2002). We hypothesize that these elevated levels of free radicals and the subsequent oxidation may play role in apoptosis induction and might also be involved in the direct membrane-damaging effects of thimerosal identified in our study.

We showed that the concentrations of thimerosal that induced toxic effects in human cortical neurons ranged from 1 to 250 μM . However, comparisons of the nuclear morphology of dying cells after incubation with higher versus lower concentrations of thimerosal demonstrate important differences. Although caspase-3 activation was detected in both high and low concentrations of thimerosal, the morphology of dying cells was different in these two situations. The cell bodies of neurons treated with higher concentrations of thimerosal (50 – 250 μM) were swollen, which is more characteristic of necrotic cell death, whereas cells treated with low concentrations (2–10 μM) were shrunken, as is typical for apoptosis (Fig. 7). Similarly, the nuclei of dying neurons treated with 250- μM thimerosal were larger in size and swollen, in contrast to the shrunken nuclei of cells treated with 2- μM thimerosal (Fig. 7). Thus, cell death occurring after incubation of neuronal cells with higher concentrations of thimerosal has features of both apoptosis (caspase-3 activation) and necrosis (cell edema and nuclei swelling). This can be explained by a direct membrane-damaging effect of thimerosal, which rapidly leads to the loss of membrane integrity and cell swelling. This process likely occurs simultaneously with apoptosis induction, the initiation of the caspase cascade, and the activation of caspase-3. At lower concentrations of thimerosal, direct membrane-damaging effects are weaker, and no swelling is observed.

Investigation of thimerosal toxicity is especially important at the present time, because this compound is used in biological products and can be administered in toxic doses either accidentally or intentionally (Ball *et al.*, 2001).

In our study, the concentrations of thimerosal that induced toxic effects ranged from 1 μM (405 $\mu\text{g/l}$) to 250 μM (101 mg/l), which is equivalent to the levels of inorganic mercury from 201 $\mu\text{g/l}$ to 50 mg/l. In clinical cases of accidental or intentional usage in high concentrations, thimerosal was administered in doses from 3 mg/kg to several hundred mg/kg (Ball *et al.*, 2001). Such doses resulted in local necrosis at the application site and severe central nervous system and kidney injury.

Much lower concentrations are reached during normal vaccination, when thimerosal-containing vaccines are used. In the case of a full series of vaccinations containing thimerosal, up to 403 μg of thimerosal (equivalent to 200 μg of mercury) are received by 6 months of age (calculated from Ball *et al.*, 2001). This results in the administration of $200/3.81 = 52 \mu\text{g/kg}$, $200/5.22 = 38 \mu\text{g/kg}$, and $200/6.27 = 32 \mu\text{g/kg}$ of mercury. These calculations utilize averages of the 5th, 50th, and 95th% weight for females at birth (2.36 kg, 3.23 kg, 3.81 kg) and at 6 months (5.25 kg, 7.21 kg, 8.73 kg) = 3.81 kg, 5.22 kg, 6.27 kg, reported by (Ball *et al.*, 2001) when used in calculating exposure limits for mercury in comparisons of various agencies guidelines.

The lowest toxic concentration of mercury contained in the thimerosal doses in our present study (201 $\mu\text{g/l}$) is less than four times higher than some of these estimated concentrations. The rapidly developing toxicity of thimerosal in low micro-molar concentrations over short time frames is of concern and suggests that additional research is necessary to estimate the effects of prolonged exposure to thimerosal in lower doses.

In this paper we demonstrated that extending the time of incubation with thimerosal from 2 to 6 h is associated with toxicity that was not seen after a shorter time of exposure. For this reason, further studies of lower concentrations and longer exposure times appear to be warranted. These results indicate that additional research is needed to more fully delineate the dose- and time-dependent toxicity of thimerosal in sub-micro-molar concentrations and suggests that toxicity may occur at even lower doses than those utilized in these experiments, with longer times of exposure. Because mercury can be retained in body organs for months to years, the study of longer incubation times is warranted. We also conclude that a proposed combination of fluorescent techniques combining the assessment of DNA, membrane damage, and active caspase-3 is useful in studying thimerosal toxicity.

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