Author

As a freshman, Lorrel Brown was encouraged by her father to try her hand at research. She found her research experience rewarding, especially working with scientists and fellow students and seeing knowledge passed on from person to person. She advises students to find a project that stimulates their curiosity, which will ensure a gratifying and enjoyable experience. Currently, Lorrel is a first-year medical student at Johns Hopkins University. She plans on combining her love of science and medicine to service as a physician who offers care in the United States and travels overseas on medical mercy missions. Lorrel also plays the piano, sings, studies ballet and jazz, volunteers, and tutors.

Key Terms

- Apoptosis
- Apoptosis-Inducing Factor (AIF)
- Cytochrome c
- Mercury
- Mitochondrial Pathway
- Neuronal Cells
- Reactive Oxygen Species (ROS)
- Thimerosal

Thimerosal Induces Programmed Cell Death of Neuronal Cells via Changes in the Mitochondrial Environment

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Abstract

Thimerosal, a preservative and anti-microbial agent used in vaccines, ophthalmic solutions, and cosmetics, is a mercury-containing compound that has raised public concern due to its potentially harmful effects. While past studies have implicated mercurial compounds in apoptosis, or programmed cell death, in human T-cells and cells of the central nervous system, no studies have examined the specific effect of thimerosal on neuronal cells, despite evidence that mercurial compounds readily cross the blood-brain barrier. This study examines whether thimerosal induces apoptosis in neuronal cells, and, if so, via which mechanism. To this end, neuronal cells were incubated in the absence and presence of thimerosal at various concentrations for various exposure times and then examined for cell viability, specific morphological changes associated with apoptosis, and changes in the mitochondrial environment. Thimerosal decreased neuronal cell viability in time- and dose-dependent trials, with 90% viability at 2 hr, decreasing to 60% viability at 24 hr (1 μM); at 5 μM thimerosal, viability decreased below 20% at 24 and 48 hr. Thimerosal caused depolarization of the mitochondrial membrane and enhanced superoxide generation. At 5 μM thimerosal, cytochrome c was released from mitochondria to the cytosol in 30% of cells at 1 hr and 85% of cells at 3 hr. Apoptosis-Inducing Factor was released in 40% and 90% of cells at 30 min and 1 hr, respectively. The results suggest that thimerosal causes apoptosis via the mitochondrial pathway and warrant continued efforts to find a replacement compound.

Faculty Mentor

Lorrel Brown's project, which looks into the effect of thimerosal to induce programmed cell death in neuronal cells, shows what an environmental hazardous agent can do in living cells. As such, it draws attention to potential adverse or toxic effects that can be induced by additives readily found in everyday hygiene and cosmetic products, and opens the door to many new and unexplored areas of research. Lorrel's fascinating work sets an example for what a UCI undergraduate student can achieve through scientific curiosity, motivation, perseverance, and diligence. However, scientific productivity is not the only reward of faculty-mentored undergraduate research. Efficient information processing, analytical and forward thinking are permanent fringe benefits.

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Introduction

Recently, concern has been raised over thimerosal, a mercury-containing anti-microbial and preservative agent used in ophthalmic solutions, cosmetics, and vaccines (Freed et al., 2002). Thimerosal has been a component of Hepatitis B, HIB, DPT, Influenza, and Meningococcal vaccines since the 1930s (Policy Statement of the AAP, 1999); it was estimated that infants could be exposed to 187.5 mg of mercury, a level exceeding safety guidelines, in their first six months due to routine immunizations (Piyasirisilp and Hemachudha, 2002). Due to concerns that thimerosal could have adverse health effects, the American Academy of Family Physicians, the American Academy of Pediatrics, the Advisory Committee on Immunization Practices, and the United States Public Health Services issued a joint statement recommending the removal of thimerosal from vaccines and the prompt development of alternative preservative and anti-microbial agents (1999).

Despite the public attention given to thimerosal, relatively little is known about its physiological effects. Thimerosal (ethyl mercury-thiosalicylate) is 49.6% ethyl mercury by weight (Mercury and Thimerosal, 2001). Mercury exists in three forms: methyl mercury, mercury vapor, and ethyl mercury (as in thimerosal). Mercury exhibits varying levels of toxicity in each form, caused in part by varying routes of exposure, doses, and sites of deposition. Because it can readily cross the blood-brain barrier, methyl mercury exerts its toxic effects primarily on the central nervous system (CNS) (Clarkson, 2002), but further studies have shown toxic effects on the immune system (Shenker et al., 1998). Previous research has established the cellular effects of methyl mercury (Nagashima et al., 1995; Shenker et al., 1999; Belletti et al., 2002; Issa et al., 2003); research in our laboratory previously examined the effects of ethyl mercury (thimerosal) in T-cells of the immune system (Makani et al., 2002).

Earlier studies have demonstrated that both methyl mercury and ethyl mercury induce apoptosis (Nagashima et al., 1995; Shenker et al., 2000; Belletti et al., 2002; Makani et al., 2002; Issa et al., 2003). Apoptosis, also known as "programmed cell death" or "cellular suicide," is an active form of cell death with particular changes in a cell's morphology and protein activity. It is characterized by cell shrinking, surface membrane blebbing, chromatin condensation, and DNA fragmentation. While apoptosis can be initiated in various manners, common effector mechanisms are induced that lead to caspase-mediated cleavage of substrates and apoptosis (Gupta, 2003).

One pathway of apoptosis, the mitochondrial pathway, can be induced by a variety of stimuli including radiation and reactive oxygen species (ROS) (Waterhouse and Green, 1999; Kroemer and Reed, 2000). Mercury has been shown to produce ROS via accumulation in the mitochondria (Nagashima et al., 1995). Specifically, studies by Shenker *et al.* (1998, 1999) have shown that methyl mercury can induce human T-cells to undergo apoptosis via ROS and changes in mitochondrial membrane potential. Our lab demonstrated that the mechanism of thimerosal-induced apoptosis in T-cells is via the mitochondrial pathway (Makani et al., 2002). However, the apoptotic effects of thimerosal, or any form of mercury, on neuronal cells have not yet been investigated. Therefore, we sought to elucidate whether thimerosal induces apoptosis in neuronal cells, and, if so, whether this apoptosis is via the mitochondrial pathway.

Materials and Methods

Cell Culture

Neuroblastoma cells (CRL-2268, ATTC, Manassas, VA) were grown at 37 °C in an atmosphere of 5% CO_2 as adherent monolayers in 24-well plates (Costar, USA) until they reached 70% confluence. The growth medium used for the culture was a 1:1 mixture of Eagle's Minimum Essential Medium with Earle's BSS and Ham's F-12 medium, supplemented with 2.5 mM L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin-glutamine.

Live-Dead Viability Assay

To examine the apoptotic effect of thimerosal on neuronal cells, test cells were incubated in the presence of thimerosal at concentrations 0.025, 1 and 5 μM for various lengths of time (2, 6, 24, and 48 hr), and control cells were incubated without thimerosal. The Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) was used to assess the viability of neuronal cells. This assay uses two dyes, calcein and ethidium homodimer (EthD-1); calcein green fluorescence (excitation/emission = 495/515 nm) indicates live cells, whereas EthD-1 red fluorescence (excitation/emission = 495/635 nm) indicates dead cells. Fluorescence was measured by a Cytofluor 4000 plate reader. Percentage of live cells was calculated from baseline calcein fluorescence; percentage of dead cells was calculated in reference to baseline EthD-1 levels.

Hoechst 33285 Staining

Apoptosis of neuronal cells was examined using Hoechst 33285 (Molecular Probes). Cells were plated onto poly-D-Lysine coated glass coverslips and incubated with thimerosal at 0, 0.025, 1, and 5 μM for 6, 24, and 48 hr. Cells were

fixed in fresh paraformaldehyde for 30 min at room temperature and incubated in permeabilization buffer (0.025% Saponin, 3% Bovine Serum Albumin in Phosphate Buffered Saline pH 7.2) with 5 µM of the nuclear-specific dye Hoechst 33285 for 15 min. After washing, coverslips were mounted with anti-fade reagent (Bio-Rad, Hercules, CA) on slides. Cells were visualized using confocal microscopy; one hundred cells per slide were scanned, and the percentage of condensed nuclei was determined.

Mitochondrial Membrane Potential

To examine depolarization of the mitochondrial membrane, cells were incubated with 0 and 5 µM thimerosal for 2 and 6 hr. During the last 30 min of thimerosal incubation, cells were treated with 5 µM of JC-1 (Molecular Probes, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide, Eugene, OR). Red fluorescence corresponds to an intact mitochondrial membrane, whereas green fluorescence indicates depolarization. Fluorescence was measured with a Cytofluor 4000 plate reader using excitation/emission = 485/530 nm for green fluorescence and excitation/emission = 495/635 nm for red fluorescence. The extent of mitochondrial membrane depolarization was calculated as the percent green JC-1 fluorescence compared to baseline levels, adjusted for background.

Reactive Oxygen Species (ROS) Formation

The fluorescent dye dihydroethidium (HE) (Molecular Probes) was used to measure production of superoxide (O_2^-) in neuronal cells following thimerosal treatment. Cells were incubated in the absence and presence of 5 µM thimerosal for 2 and 6 hr. During the last 30 min of thimerosal incubation, cells were treated with 5 µM HE. HE fluorescence was measured on a Cytofluor 4000 plate reader at excitation/emission of 485/580 nm. ROS production was determined as a percentage of baseline HE levels, adjusted for background.

Release of Cytochrome c and Apoptosis-Inducing Factor (AIF)

Cells were grown on poly-D-Lysine coated coverslips and incubated with 5 µM thimerosal for 30 min, 1 hr, and 3 hr. Cells were then loaded with a mitochondrial-specific dye Mitotracker Orange (Molecular Probes) at 150 nM during the last 15 min of thimerosal incubation. After fixing the cells in fresh paraformaldehyde for 15 min with gentle rocking at 4 °C, cells were washed six times in blocking buffer (0.05% saponin, 3% BSA in PBS pH 7.2). Then, cells were incubated overnight with anti-cytochrome c antibody (clone 6H2.B4, Pharmingen, San Diego, CA) or anti-AIF antibody (ProSci Incorporated, Poway, CA) diluted 1:200 in blocking buffer. Following six washes, cells were treated for 1 hr at room temperature with FITC-conjugated goat anti-mouse IgG antibody (Antibody Incorporated, Davis, CA) or FITC-conjugated goat anti-rabbit IgG (Oncogene, Boston, MA). After washing, coverslips were mounted onto slides with anti-fade reagent (Bio-Rad), and slides were viewed using a confocal microscope. One hundred cells per slide were scanned, and percentage cells with cytochrome c/AIF release were determined visually.

Results

Live-Dead Viability Assay

Percentages of live and dead cells were calculated according to baseline fluorescence readings (representative of three trials). Figure 1A shows the time- and dose-dependent

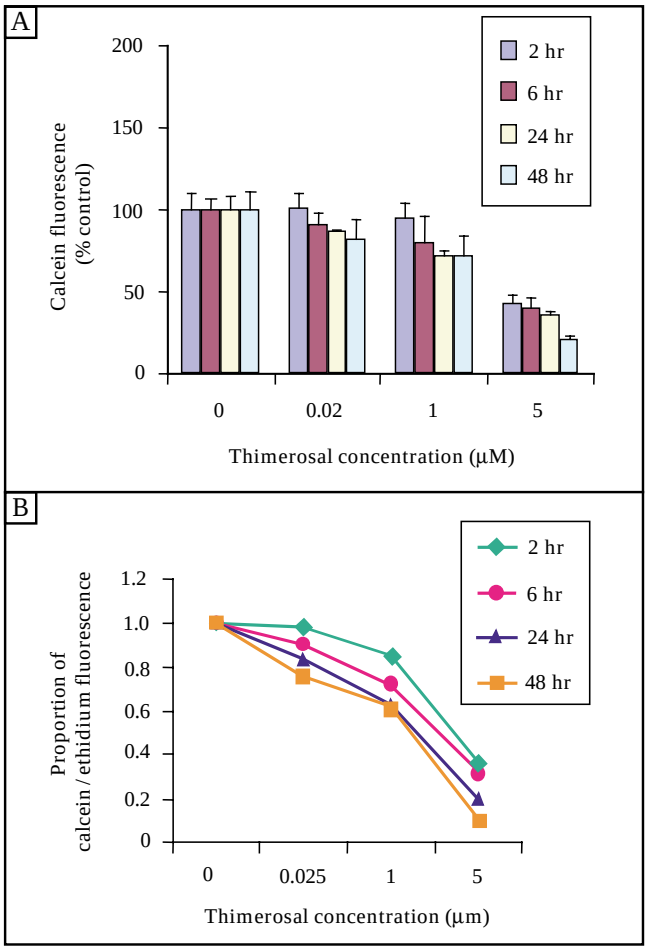

Figure 1
Thimerosal causes time- and dose-dependent decreases in calcein fluorescence (A) and in the proportion of calcein to EthD-1 fluorescence (B).

decrease in calcein fluorescence, indicating a decrease in the percentage of live cells, most notably at 5 µM. Figure 1B shows the ratio of calcein to EthD-1 fluorescence, indicating the percentage of live cells relative to dead cells. At the highest dose of thimerosal (5 µM), cell viability drops below 20%, indicating a marked decrease in viability at the 5 µM concentration. At 1 µM, there is a substantial drop in viability from 100% to 60% at both 24 and 48 hr.

Hoechst 33285 Staining

Nuclei were visualized with the blue dye Hoechst and examined for vacuolization and condensation of chromatin (Figure 2A). At 1 µM thimerosal, after 24 and 48 hr incubation, 14% of the nuclei were apoptotic. At 5 µM thimerosal, after similar incubation times, 40% of the nuclei were apoptotic. Figure 2B shows the dose- and time-dependent increase in nuclear condensation (representative of three trials).

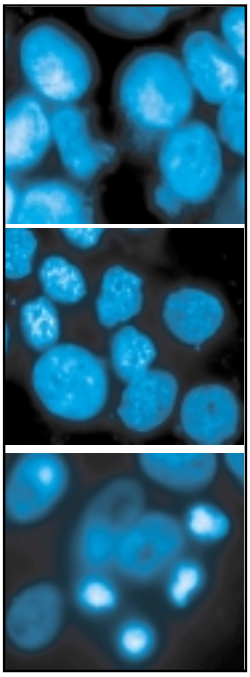

Figure 2A
Top to bottom: baseline nuclei; 0.025 µM, 48 hr treatment; 5 µM, 48 hr treatment.

Mitochondrial Membrane Potential

A shift in the JC-1 stain from red aggregates to green monomers indicates depolarization of the mitochondrial membrane. Thimerosal caused an increase in green fluorescence, calculated as a percentage of baseline values (representative of three trials), in a time- and dose-dependent fashion (Figure 3B), evidencing the increased depolarization of the mitochondrial membrane with increased thimerosal concentrations. Depolarization was evident at 2 hr but was markedly increased at 6 hr (5 µM thimerosal). Figure 3A shows a picture of predominantly red aggregates at the baseline and a shift to green fluorescence at 5 µM at 6 hr.

Reactive Oxygen Species (ROS) Formation

Production of superoxide (O_2^-) as measured by ET fluorescence was calculated as a percentage of baseline readings. Results for three trials (Figure 4) indicate that thimerosal induces production of O_2^-, most notably at 2 hr with O_2^- levels declining by 6 hr incubation.

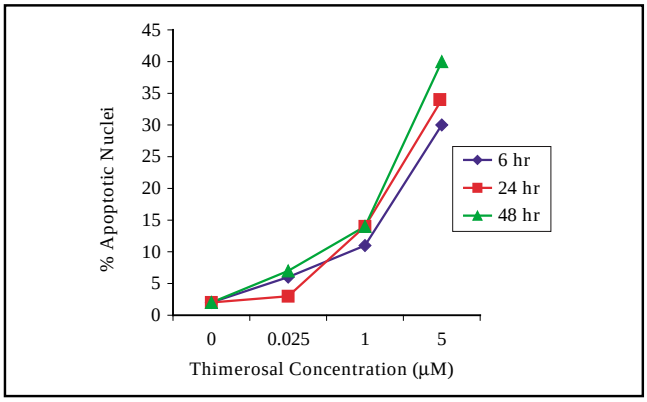

Figure 2B
Thimerosal causes an increase in apoptotic nuclei.

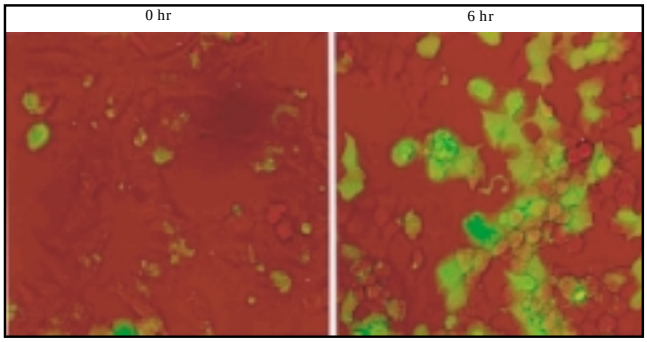

Figure 3A
Thimerosal 5 µM causes an increase in green JC-1 fluorescence.

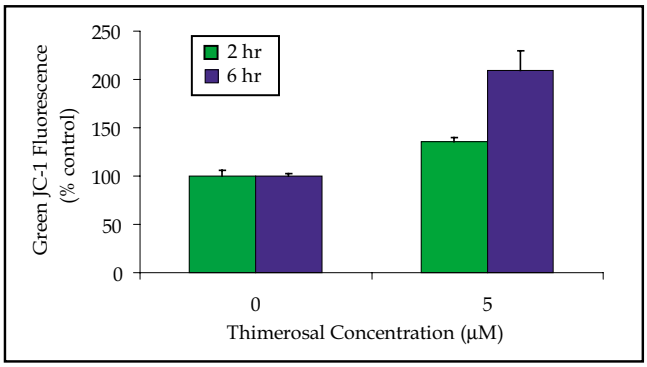

Figure 3B
Thimerosal causes a shift from red to green JC-1 fluorescence.

Release of Cytochrome c and Apoptosis-Inducing Factor (AIF)

The localization of cytochrome c and AIF within the cells was examined by fluorescence microscopy (data not shown). Figure 5A shows the location of cytochrome c (green) within the cell relative to the mitochondria (red). Figure 5B shows AIF in green and its location relative to mitochondria (red) and nuclei (blue).

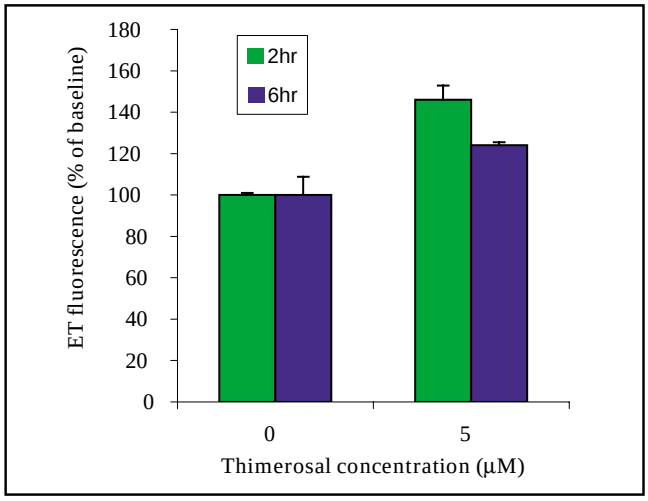

Figure 4
Thimerosal causes an increase in ROS production, predominantly at 2 hr.

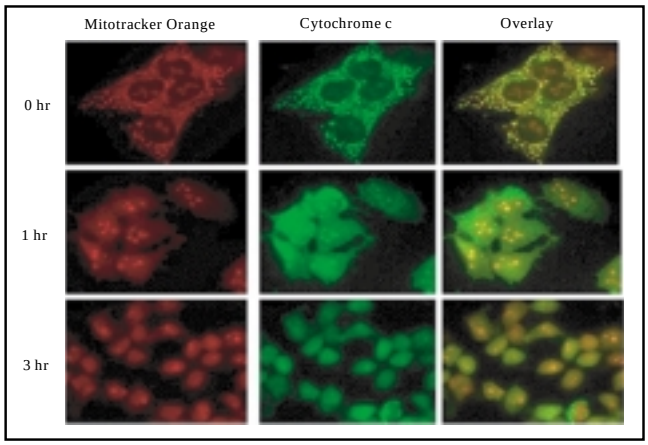

Figure 5A
Thimerosal causes release of cytochrome c from mitochondria.

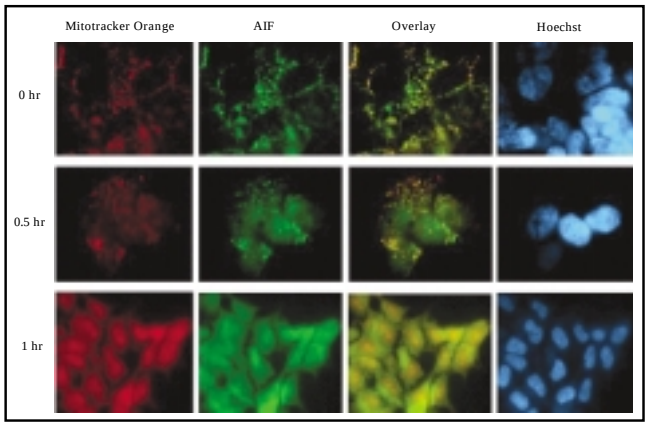

Figure 5B
Thimerosal causes release of AIF from mitochondria.

For cytochrome c, at 1 hr (5 µM thimerosal) 30% of cells showed release, whereas 85% showed release at 3 hr. The results for AIF show 40% release at 0.5 hr and 90% release at 1 hr (5 µM thimerosal). The results represent data from three trials.

Discussion

Although great concern has been raised over the compound thimerosal and its potentially hazardous effects on the central nervous and immune systems, little research has been done on the molecular and biochemical effects of thimerosal on neuronal cells. Past studies of methyl mercury, an organic yet slightly different form of mercury, have concluded that it induces apoptosis in rat cerebellar granule cells *in vivo* (Nagashima et al., 1995), human T-cells *in vitro* (Shenker et al., 1999), glioma cells *in vitro* (Belletti et al., 2002), and human oligodendroglial cells *in vitro* (Issa et al., 2003). Each of the *in vitro* studies concluded that the mechanism of apoptotic induction involves oxidative stress (production of ROS) and, in those cases in which it was specifically examined, depolarization of the mitochondrial membrane potential. The only *in vitro* study examining the molecular mechanism of thimerosal-induced apoptosis was performed in our laboratory using T-cells (Makani et al., 2002) and concluded that production of ROS and release of mitochondrial intermembrane proteins were major steps in the apoptotic pathway.

Although mercury is known to accumulate in the brain, no previous study had examined the effect of thimerosal on cells of the CNS. Therefore, we examined neuronal cells under the influence of thimerosal. We found that thimerosal causes death of neuronal cells, as evidenced by the increased ratio of EthD-1 to calcein fluorescence with increasing thimerosal exposure. The time- and dose-dependent death of neuronal cells caused by thimerosal was marked by the time- and dose-dependent decrease in the ratio of calcein to EthD-1 fluorescence (Figure 1B).

To ensure that death induced by thimerosal is due to apoptosis rather than necrosis, nuclei of treated cells were examined with Hoechst 33285 staining. Visualized nuclei displayed the characteristic morphology of apoptosis: chromatin condensation, shrinking, and vacuolization (Figure 2A). These apoptotic effects intensified with thimerosal treatments of increasing concentrations.

Depolarization of the mitochondrial membrane potential is a vital step in the apoptotic mechanism induced by mercury (Shenker et al., 1998; Belletti et al., 2002). It has also been

shown that this step occurs early in apoptosis (Shenker et al., 1999). Mitochondria are organelles consisting of an outer membrane (OM), intermembrane space, inner membrane (IM), and matrix. Enzymes of the electron transport chain, vital for oxidative respiration and production of ATP, reside in the IM and under normal physiological conditions produce an electrochemical gradient, or membrane potential. Permeabilization of the IM during apoptosis leads to a depolarization of this potential (Green and Reed, 1998; Kroemer and Reed, 2000). Therefore, we examined the effect of thimerosal on mitochondrial membrane potential using JC-1; this dye exhibits a membrane potential-dependent accumulation in the mitochondria, with red fluorescence indicating the accumulation of JC-1 aggregates in the mitochondria. A change from red to green fluorescence is due to accumulation of monomers outside the mitochondria, an indication of membrane depolarization. The shift to green fluorescence is seen mainly at 6 hr, consistent with previous observations that mitochondrial membrane potential depolarization occurs early in apoptosis (Makani et al., 2002).

During normal cellular metabolism, ROS (superoxides, peroxides, oxygen-free radicals) are created, but cells have endogenous anti-oxidants that protect them from oxidative stress (Yu, 1994). Increased levels of ROS have been shown to promote neuronal cell death and apoptosis (Keller et al., 1999; Makani et al., 2002). Therefore, we examined the production of O_2^-. Thimerosal caused an increase in O_2^-, primarily at 2 hr post-incubation.

The intermembrane space contains cytochrome c and AIF. Permeabilization of the OM allows for the release of these molecules into the cytoplasm. The subsequent translocation of AIF to the nucleus, and the conglomeration of cytochrome c with Apaf-1 (apoptotic protease-activating factor) and pro-caspase 9 form the apoptosome. Further cleavages yield activated caspases that cleave proteins to induce apoptosis (Gupta, 2003). We examined the release of cytochrome c and AIF and found that thimerosal causes release of cytochrome c and AIF from the mitochondria, with AIF release preceding cytochrome c release. These results are consistent with previously published findings that AIF release occurs upstream of cytochrome c release (Daugas et al., 2000).

Conclusion

Collectively, the results of this experiment indicate that thimerosal at relatively low doses causes apoptosis in neuronal cells via the mitochondrial pathway. To further clarify the step-wise mechanism of thimerosal-induced apoptosis, investigation of caspase protein activation is currently underway. The increased understanding of the biochemical and molecular effects of thimerosal provided by this project supports the decision of policy makers to remove thimerosal from childhood vaccines; it also helps to elucidate the detrimental effects resulting from human exposure to organic mercury compounds. Continued efforts are needed to find an anti-microbial and preservative compound to replace thimerosal in vaccines, cosmetics, and ophthalmic solutions.

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