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Thimerosal induces neuronal cell apoptosis by causing cytochrome *c* and apoptosis-inducing factor release from mitochondria

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Abstract. 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.

Introduction

Apoptosis is a physiological form of cell suicide that plays a role in embryogenesis, metamorphosis, cellular homeostasis, and as a defensive mechanism to remove infected, mutated, or damaged cells (1,2). Apoptosis is characterized by loss of cellular contact with the matrix, cytoplasmic contraction, chromatin condensation, plasma membrane blebbing, and DNA fragmentation into large and small oligosomes. Apoptosis takes place through the death receptors (3-6) and/or involvement of the mitochondrial pathway (7,8), with molecular and biochemical steps leading to the activation of common effector or executioner cysteine proteases, the caspases resulting in the cleavage of a number of nuclear and cytoplasmic substrates that culminate in apoptosis. Because of the role of apoptosis in cellular homeostasis, disorders of apoptosis result in either the accumulation of abnormal cells, leading to cancer and autoimmunity, or in the loss of cells, leading to immunodeficiency and neurodegenerative diseases (9).

There is an increasing concern throughout the world about the risks of environmental exposure to mercury, which is ubiquitously found in fish, dental amalgams, and in preservatives (10-17). One of the mercury compounds that has recently come to public attention, because of its wide usage as an antibacterial and antifungal preservative in biomedical products and vaccines, is thimerosal (10-12). Thimerosal (ethylmercury salicylate) contains 49.6% mercury by weight and is metabolized to ethylmercury and thiosalicylate (15). In the body, ethylmercury readily passes through cellular membranes and concentrates in vital tissue and organs, including the central nervous system where it can exert its toxicity over a prolonged period of time (12,16). However, the effects of thimerosal on neuronal cell functions, especially on apoptosis, are poorly understood and largely unexplored.

During the last decade, there has been a better understanding of the role of mitochondria in cell death (7,8,18-20). A number of stimuli, including chemotherapeutic agents, radiation, and stress molecules, e.g. reactive oxygen and reactive nitrogen species, appear to mediate apoptosis via mitochondrial pathway. It is known that some mercury derivatives, such as methylmercury, induce oxidative stress (21-24). In this study, in order to shed light on the claimed link between thimerosal and neurotoxicity, we investigated the effect of thimerosal on cell viability, mitochondrial microenvironment and downstream steps of apoptosis in neuronal cells. We observed that thimerosal, in nanomolar concentrations, caused cell death/apoptosis which was mediated through the mitochondria evidenced by cytochrome *c* and AIF release and activation of caspase-9.

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Abbreviations: AIF, apoptosis-inducing factor; $\Delta\Psi$, mitochondrial membrane potential; DHE, dihydroethidium; EthD-1, ethidium; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species

Key words: thimerosal, mitochondria, cytochrome *c*, apoptosis-inducing factor, bax