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Thimerosal Induces Apoptosis in a Neuroblastoma Model via the cJun N-Terminal Kinase Pathway

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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 (0–10μM) showed an increase in the phosphorylated (active) form of JNK and cJun with 5 and 10μM 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 (0–2.5μM) 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.

Key Words: **thimerosal; mercury; neurotoxicity; JNK; cJun; Bim.**

Thimerosal is an organic mercurial containing an ethylmercury moiety attached to the sulfur atom of thiosalicylate. Since the 1930's, thimerosal has been used in many products as an antiseptic and a preservative. In recent years, controversy has surrounded the use of thimerosal in vaccines as mercury is a known neurotoxin and nephrotoxin. Since the controversy began in the late 1990's, much of the thimerosal has been removed from vaccines administered to children in the United States. However, it remains in some, such as the influenza vaccine, and is added to multidose vials used in countries around the world. Studies concentrating on thimerosal-induced neurotoxicity are limited, and exposure guidelines, such as those set by the Food and Drug Administration, are based on research with methylmercury. Interestingly, some *in vitro* and *in vivo* studies suggest that ethylmercury may react differently than methylmercury (Aschner and Aschner, 1990; Harry *et al.*, 2004; Magos *et al.*, 1985). Few studies with thimerosal have focused on determining specific signaling pathways involved in neurotoxicity. Establishing these pathways may be an important step in discovering methods of alleviating toxic outcomes in patients exposed to thimerosal.

While the toxicological profile of thimerosal is still somewhat limited, the amount of information regarding thimerosal-induced toxicity is increasing. Recent studies have shown various events occurring in response to thimerosal exposure. Rat cerebellar neurons treated with thimerosal showed increases in intracellular calcium levels and decreases in glutathione levels (Ueha-Ishibashi *et al.*, 2004a, 2005). Decreased glutathione levels resulting from thimerosal exposure were also seen in rat thymocytes (Ueha-Ishibashi *et al.*, 2004b), cultured neuroblastoma cells (SH-SY5Y), and glioblastoma cells (James *et al.*, 2005). HeLa S cells treated with thimerosal showed cytoskeletal changes and activation of focal adhesion kinase, both of which were attributed to the production of reactive oxygen species (Kim *et al.*, 2002). Studies in our laboratory have begun to establish a more coordinated picture of events that occur in cells treated with thimerosal. We have shown that upon treatment with thimerosal, SK-N-SH neuroblastoma cells exhibited a time- and concentration-dependent increase in apoptotic cell death, as evidenced by increases in nuclear condensation, cytochrome *c* release, caspases 9 and 3 activation, poly(ADP-ribose) polymerase (PARP) degradation, and lactate dehydrogenase release, thus demonstrating a

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