Journal of Toxicology and Environmental Health, Part A, 70: 2092–2095, 2007

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390701551324

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Evaluation of Cytotoxicity Attributed to Thimerosal on Murine and Human Kidney Cells

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Renal inner medullary collecting duct cells (mIMCD3) and human embryonic kidney cells (HEK293) were used for cytoscreening of thimerosal and mercury chloride ($HgCl_2$). Thimerosal and $HgCl_2$ acted in a concentration-dependent manner. In mIMCD3 cells the 24-h LC_{50} values for thimerosal, thiosalicylic acid, 2,2-dithiosalicylic acid, and 2-sulfobenzoic acid were 2.9, 2200, >1000, and >10,000 μ*M*, respectively. The 24-h LC_{50} value for $HgCl_2$ in mIMCD3 cells was 40 μ*M*. In HEK293 cells, the 24-h LC_{50} value for thimerosal was 9.5 μ*M*. These data demonstrate that the higher cytotoxicity produced by thimerosal on renal cells with respect to similar compounds without Hg may be related to this metal content. The present study also establishes mIMCD3 cells as a valuable model for evaluation of cytotoxicity of nephrotoxic compounds.

Public health regulatory agencies are increasingly concerned about the impact of renal diseases produced by commercially used compounds that are nephrotoxic (Thadhani et al., 1996). In particular, renal inner medullary cells are often exposed to high concentrations of common nephrotoxic substances and also frequently are subjected to hyperosmotic and ischemic stress (Burg, 2002; Lee et al., 2002). Little is known concerning the cytotoxic effects produced by drugs and toxicants other than nonsteroidal anti-inflammatory drugs (Rocha et al., 2001) on renal inner medullary collecting duct cells (mIMCD3), which are an immortalized cell line derived from mouse renal inner medulla.

Thimerosal (ethylmercurithiosalicylic acid) is a mercury-containing preservative that has been used as an additive for vaccine and biological products for more than 70 years. Thimerosal dissociates as 49.5% ethylmercury by weight and thiosalicylic acid. High-dose, acute or chronic mercury (Hg) exposure of children and adults resulted in nephrotoxicity (Clarkson, 1993; Van Vleet & Schnellmann, 2003). Ethylmercury and thimerosal induce significantly higher Hg concentration in the kidney than in brain (Harry et al., 2004). However, thimerosal has been implicated in neuronal toxicity and autism (Geier & Geier, 2006; Kern & Jones, 2006; Kern et al., 2007).

The colorimetric cell survival assay using the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) has been widely used for measuring cell proliferation and survival (Mosmann, 1983). This assay measures the reduction of a tetrazolium component into an insoluble purple formazan product by the mitochondria of viable cell. The present study was undertaken to investigate cytotoxicity of thimerosal and its structural analogs, and inorganic Hg in mIMCD3 and human embryonic kidney (HEK293) cells.

MATERIALS AND METHODS

Cultures of mIMCD3 and HEK 293 Cells

mIMCD3 cells of passage 19 and HEK293 cells of passage 35 were used for all experiments. All reagents for cell

This study was supported by grants from the National Institute of Environmental Health Sciences Superfund Basic Research Program P42 ES04699, the National Institute of Environmental Health Sciences Center for Environmental Health Sciences P30 ES05707, and the National Institute for Diabetes and Digestive and Kidney Diseases R01-DK59470. However, it has not been subjected to funding agency review and does not necessarily reflect the views of the funding agency.

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cultures were purchased from Invitrogen (Carlsbad, CA). Cell culture medium consisted of 45% Ham's F-12, 45% Dulbecco's modified Eagle's medium, 10% fetal bovine serum (FBS), and 10 mU/ml penicillin. Additionally, 10 μg/ml streptomycin was added to the mIMCD3 cells; whilst 10% heated inactivated FBS was used instead of 10% FBS for the HEK293 cells. Cells were grown at 37ºC and 5% CO_2. Final medium osmolality of isosmotic medium was 300 ± 5 mosmol/kg medium. Final osmolality of these media was confirmed by a microosmometer (model 3300, Advanced Instruments, Norwood, MA).

Compounds

Thimerosal ($C_9H_9HgNaO_2S$) and its structural analogs including thiosalicylic acid ($HSC_6H_4CO_2H$), 2-sulfobenzoic acid anhydride ($C_7H_4O_4S$) and 2,2-dithiosalicylic acid ($S_2(C_6H_4CO_2H)_2$), and mercury chloride ($HgCl_2$) were purchased from Sigma (St. Louis, MO). Thimerosal and $HgCl_2$ were dissolved in Milli-Q water (Millipore, Bedford, MA). Thiosalicylic acid, 2-sulfobenzoic acid, and 2,2-dithiosalicylic acid were dissolved in dimethyl sulfoxide (DMSO). Final DMSO concentration did not exceed 1% in the medium. The solutions of all compounds were prepared just before use.

Determination of Cytotoxicity

Cytotoxicity was assessed using the MTT cell survival assay kit (Roche Applied Science, Indianapolis, IN). mIMCD3 and HEK293 cells were grown up, trypsinized, and seeded evenly with 100 μl of medium into each well of a flat-bottomed 96-well cell culture plate (Nalge-Nunc, Rochester, NY). Once confluent, the desired concentration of each toxicant, diluted from a 100-fold stock solution, was added to the cells and incubated in a humidified atmosphere of 5% CO_2 at 37°C, for 24 h. Controls consisted of treating cells with the same stock solution, with water or DMSO added instead of toxicants. The MTT assay was performed according to the manufacture's instruction. Briefly, 10 μ*L* final prepared MTT reagent was added into each well and cells were incubated for 4 h followed by addition of 100 μl of detergent solution into each well. After 24 h of incubation, the ratio of absorbance at 560 nm versus 750 nm was measured with a SpectraFluor Plus microplate reader (Tecan, Durham, NC). This ratio represented a measure of viable cells in each well and this ratio was normalized to controls set at 100% that were run in parallel in each 96-well plate. Each condition was repeated in eight wells and experiments were independently replicated between two and five times. The concentration at which half of the cells died after 24 h for each of the toxins tested (LC_{50}) was determined. The results were expressed as percent cell survival compared to the appropriate control. The results are also presented as mean ± SEM.

RESULTS

DMSO did not affect survival of mIMCD3 and HEK293 cells. The 24-h LC_{50} values for thimerosal, thiosalicylic acid, 2,2-dithiosalicylic acid, and 2-sulfobenzoic acid in mIMCD3 cells were 2.9, 2200, >1000, and >10,000 μ*M*, respectively and for $HgCl_2$ 40 μ*M* (Figure 1). The 24-h LC_{50} value for thimerosal in HEK293 cells presented in Figure 2 is 9.5 μ*M*, indicating that thimerosal was 3.3-fold more toxic to mIMCD3 than HEK293 cells in this study.

DISCUSSION

The results of this study demonstrated that thimerosal and $HgCl_2$ are directly toxic to mIMCD3 cells. Thimerosal displayed 24-h LC_{50} values of 2.9 μ*M* in mIMCD3 and 9.5 μ*M* in HEK293 cells. Thimerosal cytotoxicity is due to the ethylmercury content of the compound because the structural thimerosal analogs thiosalicylic acid, 2-sulfobenzoic acid, and 2,2-dithiosalicylic acid, which contain no Hg, are much less cytotoxic than thimerosal. Confirming the notion that the Hg group within thimerosal is responsible for cytotoxicity, it was found that $HgCl_2$ has a very similar LC_{50} value (40 μ*M*) in mIMCD3 cells compared to thimerosal. Our data show that thimerosal exerted significant effects on renal inner medullary cells at physiological concentrations and that mIMCD3 cells represent a useful model system for investigating mechanisms underlying thimerosal nephrotoxicity.

Ethylmercury and thimerosal induce a significantly higher Hg concentration in kidney than brain (Harry et al., 2004). Thimerosal was shown to produce renal failure in patients (Pfab et al., 1996). Cytotoxic mechanisms underlying thimerosal were reported including (1) increased intracellular Ca^{2+} concentration in several cell types (Jan et al., 2003; Ueha-Ishibashi et al., 2004), associated with induction of necrosis (Bootman et al., 2001); (2) production of apoptosis, dysfunction of proteins, and interference with ion flux (Clapham, 1995); and (3) interaction with sulfhydryl groups and oxidative stress (Kern & Jones, 2006; Kern et al, 2007). Thimerosal alters the activity of sodium channels (Evans & Bielefeldt, 2000) and Ca^{2+}-activated K^+ channels (Lang et al., 2000). It was reported that thimerosal induced DNA and membrane damage, caspase-3-dependent apoptosis (Baskin et al., 2003), activation of caspase-9 and caspase-3 (Makani et al., 2002), and activation of the cJun N-terminal kinase pathway (Herdman et al., 2006). Data showed that thimerosal and not thiosalicylic acid induced apoptosis in T cells (Makani et al., 2002).

To compare relative toxicity of thimerosal in a different cell line with our data on mIMCD3 cells, HEK293 cells were used. HEK293 cells showed a cellular toxicity profile to thimerosal comparable with that of mIMCD3 cells but did not appear quite as sensitive to thimerosal. In summary, the present study found that thimerosal is highly toxic to mouse and human kidney cells. Thimerosal cytotoxicity was attributed to Hg

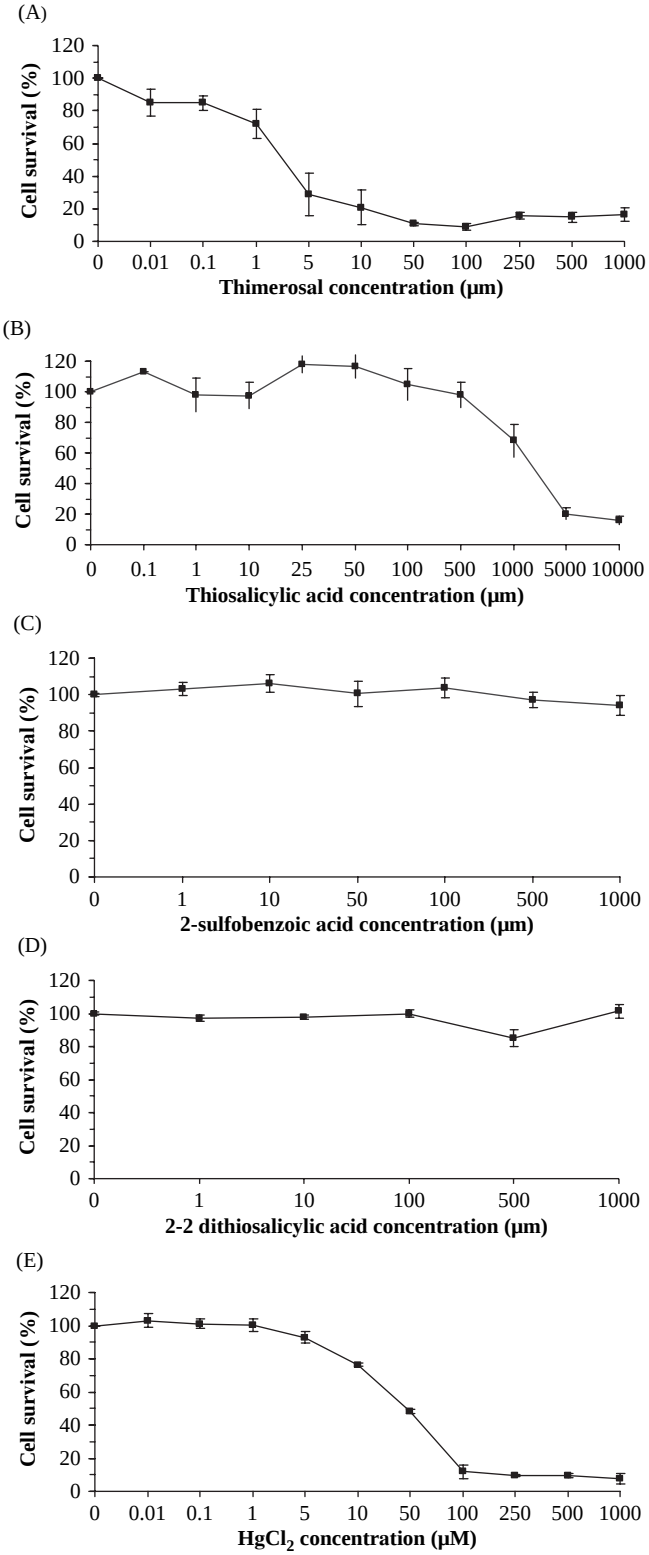

FIG. 1. Cytotoxicity due to (A) thimerosal and its non-Hg structural relatives and metabolites, (B) thiosalicylic acid, (C) 2-sulfobenzoic acid, (D) 2,2-dithiosalicylic acid, and (E) $HgCl_2$ in mIMCD3 cells in normal isosmotic (300 mOsm/kg) medium. Data are expressed as percent cell survival compared to control (2–5 independent experiments).

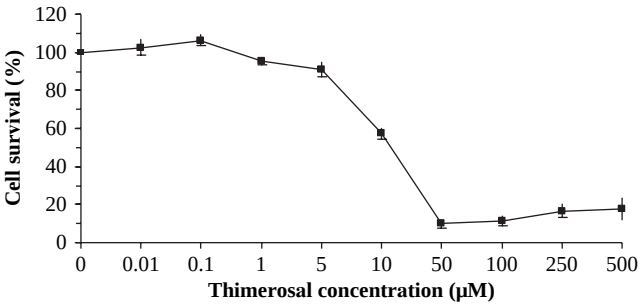

FIG. 2. Cytotoxicity attributed to thimerosal in HEK293 cells in normal isosmotic (300 mosmol/kg) medium. Data are expressed as percent cell survival compared to control (two independent experiments).

content. Furthermore, mIMCD3 cells were shown to be a valuable model for studying as the mechanism underlying thimerosal toxicity.

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