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In vitro Effects of Thimerosal and Its Metabolites on Cell Proliferation Kinetics and micronuclei frequency of Stimulated Human Lymphocytes (-S9/+S9)

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ABSTRACT

Thimerosal is an ethylmercury-containing preservative in vaccines and biomedical preparations. Little is known about the genotoxicity reactions of thimerosal in human peripheral blood lymphocytes in vitro. In the study, short-term thimerosal toxicity was investigated in cultured human peripheral blood lymphocytes under conditions with and without S9 fraction. Cytokinesis-Block Micronucleus Test is a useful technique for the assessment of genotoxicity. Cultured human peripheral blood lymphocyte cells were incubated with 0.2μg/ml-0.6μg/ml concentrations of thimerosal for 72 h at 37^{o}C under conditions without S9 fraction. Cultured human peripheral blood lymphocyte cells were incubated with 0.2μg/ml-0.6μg/ml concentrations of thimerosal for 3 h at 37^{o}C under conditions with S9 fraction. Thimerosal induced the formation of micronuclei (MN) in a dose-dependent manner in the cytokinesis-blocked lymphocytes under conditions both with and without S9 fraction. Besides, thimerosal significantly decreased the cytokinesis block proliferation index in all the doses when compared with negative control, except at the dose of 0.2 µg/mL compared with negative control.

Keywords: Thimerosal, Micronucleus, human lymphocytes, cytokinesis block proliferation index.

1. INTRODUCTION

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% (30–100μg/ml) (Ball *et al*., 2001). Thimerosal (ethyl mercury-thiosalicylate) is 49, 6% ethyl mercury by weight. In the body, ethylmercury can be converted to inorganic mercury. 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).

Some recent in vitro studies show that certain concentrations of thimerosal have decreased cellular viability in human neurons and fibroblasts. For example, Baskin et al. (2003) noted an increase in membrane permeability to DAPI dye as early as 2 h after incubation of human cortical neurons and fibroblasts with 250 μM thimerosal. A 6 h incubation resulted in

membrane damage (loss of DAPI dye exclusion), DNA breaks, and apoptosis as indicated by morphology and caspase-3 activation (Baskin et al., 2003).

Thimerosal has been recognized by the California Environmental Protection Agency, Office of Environmental Health Hazard Assessment as a developmental toxin, meaning that it can cause birth defects, low birth weight, biological dysfunctions, or psychological or behavior deficits that become manifest as the child grows, and that maternal exposure during pregnancy can disrupt the development or even cause the death of the fetus. Despite the public attention given to thimerosal, relatively little is known about its genotoxicity effects on the human peripheral blood lymphocytes in in vitro. Thimerosal genotoxicity was shown on the different cell types in numerous studies.

In vitro assays for defining toxicological effects are becoming increasingly important as the burden of necessary investigations increases. Micronuclei (MN) in peripheral blood lymphocytes are among the cytogenetic markers most widely used for the detection of early biological effects induced by DNA-damaging agents (Fenech and Morley 1985; Çelik 2006). MN can be formed from acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division. Both clastogenic and aneugenic effects can be determined with the MN test. Furthermore, an increased MN frequency in peripheral blood lymphocytes implies DNA damage risk in human in vivo (Kirsch-Volders et al. 1997).

The first developments and the standardization of the in vitro micronucleus test were carried out mainly using human lymphocytes (Fenech and Morley 1985; Van Hummelen, et. al, 1990; Van Hummelen et. al. 1992; Fenech 1993), an available and well studied cell type widely used for biomonitoring studies and biomedical surveys (Van Hummelen, et. al, 1994; Buchet et. al., 1995). Much experience has been accumulated on the in vitro micronucleus test using human lymphocytes and it has proved a useful test, which is of benefit when performing genotoxicity evaluations of clastogenicity . In addition to clastogenicity, most studies showing the ability of this test system to detect aneugens were carried out on human lymphocytes (Fenech and Morley 1985; Van Hummelen, et. al, 1990; Eastmond and Pinkel 1990; Migliore and Nieri, 1991).

The present study was designed to describe and explain the possible genotoxic effects of thimerosal on human peripheral blood lymphocytes cultured in vitro under conditions with and without S9 fraction by using Cytokinesis Block micronucleus test (CBMN).

2. MATERIAL and METHOD

2.1. Donors and collection of blood samples

The study was carried out by using blood samples from three healthy, non-smoking male donors, aged 20, 21 and 23 years. Approximately, 10 ml of blood was collected, by venipuncture, into syringes containing sodium heparin as anticoagulant. Blood was taken the same day of the initiation of the experiment between 9.00 and 9.30 a.m. to minimize possible confounding effects of dietary factors.

2.2. Test chemicals

Thimerosal (CAS No. 54–64–8) was obtained from Sigma (Figure 1). Mitomycin C (MMC, CAS No: 50–07-7, Kyowa). Methanol, acetic acid, potassium chloride, sodium chloride, tri-sodium citrate-2-hydrate, di-sodium hydrogen phosphate-2-hydrate, potassium dihydrogen phosphate and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine was purchased from Fluka (Buchs, Switzerland). Phytohaemagglutinin (PHA, M form) was

obtained from Gibco BRL Life Technologies (Paisley, UK) and prepared according to supplier's instructions. Heparin was purchased from Braun (Melsungen, Germany). Cytochalasin B was obtained from Sigma. In the cultures without metabolic activation, the positive control was MMC at 2μg/ml for treatments. For cultures with metabolic activation, cyclophosphamide (CP, CAS No. 6055-19-2, Sigma) was used as positive control.

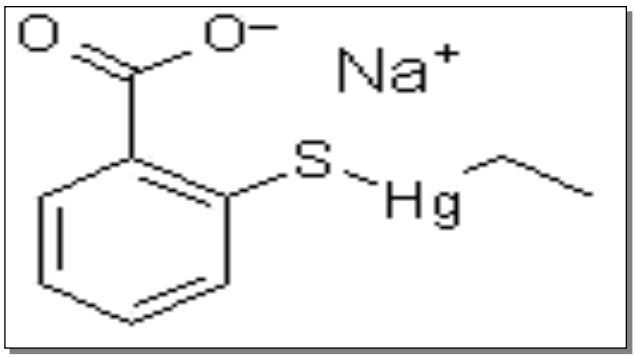

Figure 1. The Chemical structure of Thimerosal

2.3. Dose Selection

The dose range was selected according to concentrations (30-100 μg/ml) of TH in vaccines (Ball et al., 2001). TH was used at final concentration of 0.2, 0.4 and 0.6 μg/ml. In this study, "the highest dose of TH" 0.6 μg/ml was used as 1/50 of concentration in vaccines (30μg/ml).

2.4. Metabolic activation

S9 from (Cat No: 452591), "BD Biosciences" was used as metabolic activation system. The rats used to produce the S9 were not given arochlor, phenobarbitol, or other chemicals. The S9 mix freshly prepared, consisted of 1ml of S9, 0.33 ml of 1M KCl, 0.32 ml of 0.25M MgCl2·6 H2O, 0.25 ml of 0.2M glucose-6-phosphate, 1ml of 0.04M NADP, 2.10 ml of distilled water and 5 ml of phosphate buffer (pH 7.4).

2.5. Cytogenetic Protocols

2.5.1. Lymphocyte Culture and MN Analysis

Blood samples were taken from three different healthy young volunteers (average age 23 years) by venipuncture using heparinized vacutainers. Lymphocytes cultures were set up in the laboratory at a sterilized place and prepared according to the technique described by Fenech (1993) with slight modifications. Heparinized whole blood (0.8 mL) was added to 4, 5 mL of culture medium RPMI 1640 (Sigma), supplemented to 20% with fetal calf serum (Sigma), with 0.2 mL phytohemagglutinin (Sigma), and with antibiotics (10,000 IU/mL penicillin and 10,000 IU/mL streptomycin). Thimerosal was diluted in distilled water to yield at final concentrations of 0.2-0.6 µg/mL at from beginning of culture and was sterilized by filtration. A final concentration of 6 µg/mL of Cytochalasin B was added to cultures 44 h later to arrest cytokinesis. At 72 h of incubation, the cultures were harvested by centrifugation at 2000 rpm for 10 min. Then, to eliminate red cell and to keep the cytoplasm, the cell pellet was treated with a hypotonic solution (4–5 min 0.075 M KCl at 37°C). Cells were centrifuged, and Carnoy's fixative (methanol: acetic acid, 3:1, v/v) solution was freshly added. This fixation step was repeated five times. Next, cell pellets were resuspended in a small volume of fixative

solution and dropped onto clean, cold slides. The slides were stained with 10% Giemsa dye solution.

In general, the same procedures were used for the assays conducted with and without metabolic activation. Nevertheless, in the case of cultures with metabolic activation, 24 h after the initiation of cultures, 0.5 ml of S9 mix were added together with the test agent (0.2, 0.4, 0.6 μg/ml of thimerosal). After an incubation period of 3 h at 37 ◦C, the test chemical and S9 mix were removed from the culture. The pellet of lymphocytes was washed twice with 5ml of RPMI 1640 medium and resuspended in complete medium and, after that, the cultures were incubated until the entire period of 72 h at 37 ◦C.

2.5.2. Scoring Criteria for Micronuclei and Cytokinesis-Block Proliferation Index

In order to determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of MN in lymphocytes (MNL), a total of 2000 binucleated cells with well-preserved cytoplasm were scored exactly for each subject and each dose on coded slides. MN were accepted only when (i) they were separated from the main nuclei, but included within the corresponding cytoplasm, (ii) they had a chromatin material similar to that of the main nuclei, (iii), and they were coplanar to the main nuclei. In the MN study, toxicity was evaluated by classifying cells according to the number of nuclei. The well-known cytotoxicity index was used: an index for measuring the cell proliferation kinetics, called the cytokinesis-block proliferation index (CBPI) (Fenech and Morley, 1985), which was calculated following the expression:

$$CBPI= MI +2\ MII +3(MIII, MIV) /100$$

Where MI–MIV represents the numbers of cells with one to four nuclei, respectively, and MIII and MIV are equally considered to be in their third cell cycle. As previously demonstrated, the CBPI is an accurate and biologically relevant index in detecting cellular toxicity or cell-cycle delay (Fenech and Morley, 1985)

2.6. Statistical Analysis

Data were compared by one-way analysis of variance. Statistical analysis was performed using the SPSS for Windows 9.05 package program. Multiple comparisons were performed by least significant difference (LSD) test. $P < 0.05$ was considered as level of significance.

3. RESULTS

A representative photomicrograph of micronucleated cell from thimerosal-treated human peripheral blood lymphocytes is shown in Figure 2.

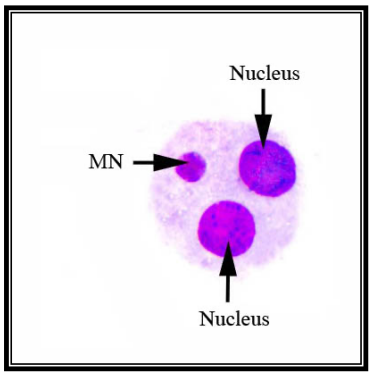

Figure 2. Giemsa-stained binucleated lymphocytes in human peripheral blood treated with thimerosal. Arrows indicate micronucleus(MN) and main nucleus.

Table 1 shows the results of the genotoxic evaluation of thimerosal in human blood lymphocytes by use of micronucleus test under conditoions with and without S9 activation. The results obtained for the MN assay after treatment lasting 72 h in human blood peripheral lymphocyte cultures set up from three healthy donors (1, 2, 3 mean age 21,3 years) indicate that TH has cytogenetic activity, inducing dose-dependent increase in the frequency of MN in three donors. There are statistically significant differences from the treated group compared with control (Figure 3). The increase in MN frequency shows that TH may induce genotoxic damages in peripheral blood lymphocytes under conditions both with S9 and without S9 fraction (Table 1).

With respect to the cytotoxic effects of the test compound, measured as a cell-cycle delay (CBPI), a dose-dependent reduction in cell proliferation was also observed in three donors, reaching statistical significance at all the doses of TH compared with control cultures ($p< 0.001$). A significant difference was observed between all the doses of TH, except at the dose of 0,2 µg/mL compared with negative control (Figure 4). Our results indicate that TH can exert cytotoxic or cytostatic effects in human peripheral blood lymphocyte cells under *in vitro* conditions (Table 1).

Table 1. The frequency of MN and CBPI values in human blood lymphocyte cultures treated with thimerosal (TH) with and without S9 fraction in vitro.

Treatments	Donor 1		Donor 2		Donor 3		Mean±SE	
	MN (‰)	CBPI	MN (‰)	CBPI	MN (‰)	CBPI	MN (‰)	CBPI (%)
NC	1.30	2.04	2.00	2.00	1.60	2.00	1.63± 0,20	2.01±0,01
NC+S9	1.60	2.08	2.00	2.01	2.00	2.01	1.86± 0,13	2.03±0,02
0,2	4.30	2.00	4.00	2.00	4.30	1.90	4.20±0,10*	1.96±0,03
0,2+S9	4.30	2.00	4.70	1.96	4.70	2.01	4.56±0,13**	1.99±0,01
0,4	5.60	1.85	5.00	1.84	5.00	1.85	5.20±0,20*	1.84±0,00*
0,4+S9	5.30	1.82	5.80	1.82	5.00	1.86	5.36±0,23**	1.83±0,01**
0,6	6.00	1.75	5.30	1.76	5.00	1.75	5.43±0,29*	1.75±0,00*
0,6+S9	5.66	1.72	6.00	1.72	7.66	1.74	6.44±0,61**	1.72±0,00**
PC(MMC, 2µg/ml)	10.30	1.51	10.70	1.46	8.30	1.46	9.76±0,74*	1.47±0,01*
PC(CP, 1.4µg/ml)+S9	10.33	1.46	9.66	1.51	10.33	1.46	10.10±0,22**	1.47±0,01**

**p<0.001, compared with negative control in condition without S9, **p<0.001, compared with negative control in condition with S9.MN: Micronucleus; CBPI: Cytokinesis Block proliferation index; SE: Standart Error; NC: Negative Control; PC: positive Control; MMC: Mitomycin C; CP: Cyclophosphamide.*

4. DISCUSSION

A study on thimerosal reveals that this compound is potent to cause genotoxic damage in human lymphocytes in the absence of S9 mix as well as in the presence of S9 mix at the doses selected. Our data indicates that thimerosal is genotoxic to human peripheral blood lymphocytes when applied in concentrations (0,2-0,6µg/ml) in in vitro under conditions with and without S9 fraction. Toxicity occurred at all the doses of thimerosal except at the dose of 0.2 µg/mL compared with negative control. A toxicity related increase of the MN frequencies was observed at all the doses in this study. MN assay is a useful analyzing method to determine the genetic damage in short-term genotoxicity tests.

There is little evidence about genotoxicity of thimerosal in peripheral blood lymphocytes in vitro with or without S9. However, thimerosal exerts genotoxic action on primary human lymphocytes at concentrations ranging from 50 to 500 ng/ml (Westphal et al., 2003). In some studies, it was reported that thimerosal was genotoxic in several cell types, such as neurons, fibroblasts by researchers. Seelbach et al. (1993) used modified micronucleus assay with V79 cells for detection of aneugenic effects and reported that thimerosal was genotoxic. Baskin et. al. (2003) demonstrated that thimerosal in micromolar concentrations rapidly induce membrane and DNA damage and initiate caspase-3–dependent apoptosis in human neurons and fibroblasts.

It is determined that thimerosal lead to oxidative damage via ROS in different cells by many researchers. Kiffe et al. (2003) investigated the effects of thimerosal at concentrations ranging from 0.25 to 4 µg/ml by using standart comet assay and measured viability of cells. Since the effect was concentration-dependent, the increase in tail length and tail moment at 2 µg /ml is considered to be biologically significant. They reported that the chemical structure (the presence of a carboxyl group and a thio group) suggests that thimerosal might be able to form cross-links.

Ueha-Ishibashi et al. (2004) investigated effects of thimerosal, a preservative in vaccines, on intracellular Ca2+ concentration of rat cerebellar neurons. They found that thimerosal exerts some cytotoxic actions on cerebellar granule neurons dissociated from 2-week-old rats. Thimerosal is metabolized to ethylmercury and thiosalicylate under the in vivo condition. They reported that both thimerosal and methylmercury increased the $[Ca^{2+}]i$ and oxidative stress in cerebellar granule cells. Pichichero et al. (2002) reported the estimate half-life of ethylmercury is 7 day and the concentrations of mercury in the blood of infants receiving vaccines containing thimerosal do not exceed the safe limits for mercury. Magos et al. (1985) found the ethylmercury is less neurotoxic than methylmercury in adult male and female rats.

Many researhers studied the immnosupressive effect of Hg and MeHg. 100 mg HgCl2/L drinking water (ca. 1800 µg Hg/kg body weight [bw]/day) for 28 days did not affect the T and B-cell populations in the bone marrow and spleen in outbred CD-1 mice (Brunet et al. 1993). In contrast, MeHg doses of 280–600 µg Hg/ kg bw/day reduced the immune response in outbred mice (Blakley et al., 1980; Thompson et al. 1998). A similar dose of Hg (590 µg Hg/kg bw/day), given as thimerosal, caused 65% reduction in the number of T- and B-cells in the spleen during the first weeks after onset of treatment (Havarinasab et al 2005). As indicated, these observations clearly demonstrate that both methyl mercury and thimerosal (ethyl mercury) are much more potent immunosuppressive substances than inorganic Hg.

It is explained that there is the suppressive effects of mercurials on the immune system in in vitro and in vivo studies by many researchers. Daum et al (1993) reported that mercuric chloride affects the function of cells and organs at lower doses. For an example, mitogen induced lymphocyte proliferation is inhibited at 10^{-7} M. Shenker et al. (1992) found that the

LD_{50} for MeHg in lymphocytes is 10^{-6} M, but lymphocyte functions are inhibited already at a dose of 10^{-7} –10^{-8} M. In addition, the LD_{50} is 2.5-fold higher in T- as compared with B-cells, and a 10- fold higher concentration is needed to inhibit mitogen induced T-cell proliferation as compared with B-cell proliferation. Baskın et al. (2003) indicated that thimerosal is toxic to human neurons and fibroblasts if applied in micromolar concentrations (1–250 M). Assesment of thimerosal toxicity is especially important at the present time, because this compound has been used in biological products and can be administered in toxic doses either accidentally or intentionally. The molecular mechanism of mercury genotoxicity is explained in four different ways.. When mercury is taken into the cell (1), it can induce the formation of reactive oxygen species either by interacting directly with DNA or indirectly by inducing a conformational change in special proteins that maintain the structure of DNA (DNA repair enzymes, proteins of microtubules). Mercury compounds may be also able to bind directly to: (2) DNA molecules, forming mercury species-DNA adducts, (3) "zinc fingers" core of DNA repair enzymes (white large arrow), affecting their activity and (4) microtubules, avoiding mitotic spindle formation and chromosome segregation (Dórea, 2019).

Possible carcinogenic effects of thimerosal were investigated in some studies. Mason et al. (1971) investigated the toxic effects of thimerosal in Fisher 344 rat. They applied by subcutaneous injection the thimerosal at doses of 0.1, 0.3, and 1.0 mg/kg body weight in 250 µl physiological saline, twice weekly for 12 months. A non-significant increase of tumours was found in the high-dose thimerosal group. As indicated by numerous researchers, inorganic and organic mercurials, including thimerosal, have been shown to increase reactive oxygen species (ROS) and induce a state of ''oxidative stress'' in numerous cell types; however, it is unknown whether this is a direct effect of the metal. Studies have shown that thimerosal causes release of calcium from intracellular stores followed by an influx of extracellular calcium (Gericke et al., 1993; Elferink, 1999), which can lead to an increase in reactive oxygen and nitrogen species (RNS) production in the mitochondria. Both ROS and RNS have been shown to cause apoptotic cell death (McGowan et al., 1996; Szabo, 1996; Cai and Jones, 1998; Chung et al., 2001). ROS and RNS can directly interact with macromolecules such as proteins and nucleic acids, leading to perturbations of vital cellular functions which may result in cell death.

In conclusion, thimerosal induced strong genotoxic effects in the cytochalasin B block in vitro micronucleus test in human lymphocytes at 0.2-0.6 µg/ml concentrations in vitro under conditions with S9 and without S9 and affected the proliferation of lymphocytes by decreasing cytokinesis Block proliferation index.

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