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GENOTOXICITY

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Thimerosal induces micronuclei in the cytochalasin B block micronucleus test with human lymphocytes

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Abstract Thimerosal is a widely used preservative in health care products, especially in vaccines. Due to possible adverse health effects, investigations on its metabolism and toxicity are urgently needed. An in vivo study on chronic toxicity of thimerosal in rats was inconclusive and reports on genotoxic effects in various in vitro systems were contradictory. Therefore, we reinvestigated thimerosal in the cytochalasin B block micronucleus test. Glutathione S-transferases were proposed to be involved in the detoxification of thimerosal or its decomposition products. Since the outcome of genotoxicity studies can be dependent on the metabolic competence of the cells used, we were additionally interested whether polymorphisms of glutathione S-transferases (GSTM1, GSTT1, or GSTP1) may influence the results of the micronucleus test with primary human lymphocytes. Blood samples of six healthy donors of different glutathione S-transferase genotypes were included in the study. At least two independent experiments were performed for each blood donor. Significant induction of micronuclei was seen at concentrations between 0.05–0.5 μg/ml in 14 out of 16 experiments. Thus, genotoxic effects were seen even at concentrations which can occur at the injection site. Toxicity and toxicity-related elevation of micronuclei was seen at and above 0.6 μg/ml thimerosal. Marked individual and intraindividual variations in the in vitro response to thimerosal among the different blood donors occurred. However, there was no association observed with any of the glutathione S-transferase polymorphism investigated. In conclusion, thimerosal is genotoxic in the cytochalasin B block micronucleus test with human lymphocytes. These data raise some concern on the widespread use of thimerosal.

Keywords Thimerosal · Cytokinesis-block micronucleus assay · Glutathione S-transferase

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Introduction

Thimerosal {sodium ethyl[2-mercaptobenzoato(2–)-*O*, *S*]mercurate(1–), CAS 54-64-8}, is used as a preservative in medical products, especially in hepatitis B vaccines. The discussion on toxic effects of thimerosal is mainly focussed on its mercury content (Ball et al. 2001). In addition, the substance is known to be a contact sensitiser (Schnuch et al. 1998). Possible carcinogenic effects were investigated in one study on the chronic toxicity of thimerosal in Fischer 344 rats (Mason et al. 1971). However, this study does not meet the requirements of the current guidelines and does not rule out a possible carcinogenic effect of thimerosal.

In addition, there were reports on genotoxic effects of thimerosal in vivo. A weak but significant increase in micronuclei and chromosome aberrations was seen in male Swiss CD-1 mice at doses between 10 and 20 mg/kg (Marrazzini et al. 1994); another study using male and female (102/E1×C3H/E1)F_1 mice and Swiss albino mice reported negative results (Adler et al. 1991).

Reports on genotoxic effects in in vitro systems were contradictory. According to the acceptance criteria outlined by the GUM (German Section of the European Environmental Mutagen Society) working group on the evaluation of published data of the in vitro micronucleus test, only two valid reports on the effects of thimerosal in this test system were available (Miller et al. 1998). A weak but significant induction of micronuclei was found at concentrations between 0.01 and 0.16 μg/ml in two out of three experiments with human lymphocytes from two donors (Migliore and Nieri 1991). A significant elevation of micronuclei in V79 cells was induced by

1.0 μg/ml thimerosal; however, only one experiment was performed (Seelbach et al. 1993).

Other reports, which did not meet the acceptance criteria of the GUM working group, showed negative results. Thimerosal did not induce micronuclei in primary human lymphocytes (van Hummelen and Kirsch-Volders 1992) or in the micronucleus assay with CREST staining with ENR and LEO human fibroblasts (Bonatti et al. 1992). Thimerosal was not genotoxic in an embryonic hamster cell line (Antoccia et al. 1991) and equivocal results were obtained in the cytochalasin B micronucleus assay with Chinese hamster Luc2 cells (Lynch and Parry 1993). However, these cell lines are rarely used and we found no historical data in the literature which would allow an estimation of their sensitivity.

There were also conflicting reports on the genotoxic effects in other in vitro systems. Thimerosal gave negative results in the Ames test (Zeiger et al. 1987). Data from various test systems on the aneuploidy inducing potential of thimerosal (including the micronucleus test) were contradictory (Parry and Sors 1993). Mutagenic effects of thimerosal in combination with UVA irradiation were reported in the thymidine kinase assay using mouse lymphoma L5178Y cells. Since thimerosal does not absorb UVA light, the relevance of this finding is unclear (Withrow et al. 1989). However, similar results were obtained in the *E. coli* DNA polymerase A assay. These results were achieved with thimerosal and illumination with visible light (Lovely et al. 1992).

Data from various test systems on the aneuploidy inducing potential of thimerosal were contradictory (Natarajan 1993). However, the induction of chromosome aberrations and micronuclei in male Swiss CD-1 mice by thimerosal rather pointed towards a clastogenic effect of thimerosal (Marrazzini et al. 1994).

Due to the contradictory results of earlier studies on the genotoxic effects of thimerosal, we reinvestigated the substance in the cytochalasin B block micronucleus test in primary human lymphocytes. Since glutathione and glutathione S-transferases have been discussed to be protective against the toxic effects of thimerosal (Dierickx 1985, Santucci et al. 1998, Westphal et al. 2000) and cell lines can differ very much regarding their sensitivity towards the toxic effects of certain substrates (Tew 1994), we were interested in whether the glutathione S-transferase polymorphisms (GSTM1, GSTT1, and GSTP1) may influence the outcome of the in vitro micronucleus test.

Materials and methods

The study was approved by the Institutional Review Board of the Medical Faculty of the Georg-August-University, Göttingen. Written informed consent was obtained from all participants.

Micronucleus test

The cytokinesis block micronucleus assay was carried out according to Fenech (1993). Human blood was collected by venipuncture in heparinised tubes, diluted 1:1 in phosphate-buffered saline (PBS) and separated by a FICOLL gradient (Biochrom KG, Berlin, Germany) for isolation of lymphocytes. Approximately 1.2×10^6 cells were seeded in 2.5 ml RPMI medium (ICN, Eschwege, Germany). Cell viability was examined by the Trypane Blue Exclusion Test (Merck, Darmstadt, Germany). Lymphocyte stimulation was done by addition of 5 μg/ml phytohemagglutinin (Biochrom KG, Berlin, Germany) and cultivation at 37 °C, 100% humidity, and 5% CO_2 for 68 h. Up to 0.6 μg thimerosal/ml water (minimum 97% purity) was added 24 h after the cultures were started. The thimerosal solution was prepared freshly for each experiment, unless stated otherwise. Cell division was blocked 44 h after the cultures were started by addition of 4.5 μg/ml cytochalasin B. Mitomycin C served as positive control. Thimerosal, cytochalasin B, and mitomycin C were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Cells were sampled 28 h after addition of cytochalasin B by centrifugation for 5 min at 175*g* and 20 °C. Hypotonic treatment consisted of careful resuspension of the cells in 5 ml hypotonic saline (0.07 M KCl/0.15 M NaCl). Immediately after addition of the hypotonic solution, the cells were collected for 5 min at 175*g* and 20 °C. For fixation, the pellet was resuspended in 3 ml ice-cold methanol/acetic acid (4:1). Cell fixation was performed twice. Finally the cells were pelleted for 5 min at 4 °C and 175*g* and transferred onto ice-cold degreased slides. The slides were randomised and subsequently stained with 3% Giemsa (Gurr/Promochem GmbH, Wesel, Germany) solution for 15 min.

Blood from six donors with different glutathione S-transferase M1 and T1 genotypes were used. For each donor at least two independent experiments were performed and at least six concentrations were examined. The maximum concentrations of the test substance were determined by evaluation of the nuclear division index (NDI) according to Eastmond and Tucker (1989). Cell scoring was done according to the criteria outlined by Fenech (1993). In addition, only binucleated cells and cells not containing more than four micronuclei with preserved cytoplasm were included in the evaluation. Acceptance criteria for a genotoxic effect were: micronuclei exceeding the control significantly, and stable pH. Only samples with at least 1000 binucleated cells were included. More than one succeeding concentration yielding cell counts <1000 binucleated cells or an NDI$\geq$1.1 were regarded as toxic effect.

Genotyping for GSTM, GSTT1, and GSTP1

Blood was collected in EDTA tubes. Extraction of DNA from the buffy coat was performed by a salting out procedure (Miller et al. 1988): lymphocytes were collected at 400*g* and 4 °C, and lysed in 50 mM TrisCl pH 8.0, 20 mM EDTA pH 8.0, and 2% SDS. Protein was digested with Proteinase K (Amresco, Solon, Ohio, USA) (at least 6 h at 56 °C) and pelleted with 6 M NaCl following centrifugation at 5000*g* for 20 min. DNA was precipitated with 1.5 times the volume 100% ethanol. DNA yields were between 0.1 and 1 μg/μl.

Homozygous deletions of *GSTM1* and *GSTT1* were characterized simultaneously with *β*-globin as internal standard (Westphal et al. 2000). The sequences of the primers for the determination of *GSTM1* were obtained from Bell et al. (1992). Determination of *GSTT1* was done using the primer pair reported by Pemble and co-workers (1994). Each PCR contained ca. 200 ng of genomic DNA, 11.25 pmol dNTPs (Amresco, Solon, Ohio, USA), 20 pmol of each primer (Eurogentec, Seraing, Belgium), 1 U *Taq* polymerase (Promega, Mannheim, Germany), and Taq buffer in a final volume of 30 μl. The samples were denaturated for 4 min at 94 °C, and subsequently amplified by 31 cycles at 94 °C, 66 °C, and 72 °C for 1 min each, without a final extension time. PCR products were separated on 2% agarose gel (molecular biology grade, Eurogentec). The *GSTP1* polymorphism resulting in an Ile→Val substitution at position 104 in exon 5 was analysed by the method of Watson et al. (1998), whereas the Ala→Val substitution at position 115 in exon 6 was analysed as described by Saarikoski

et al. (1998). Restriction endonucleases were obtained from New England Biolabs (Frankfurt a.M., Germany).

Statistical analysis

Fisher's exact test, two-sided, was performed with commercially available software (STATISTICA, version 6.0).

Results

Thimerosal induced a significant increase in micronuclei in primary human lymphocytes in 14 out of 16 experiments at non-toxic concentrations between 0.05–0.5 μg/ml. There were pronounced increases in micronuclei in several experiments (Table 1). In six experiments this increase was dose-dependent, at least two consecutive concentrations having significantly increased aberration frequencies, according to the criteria outlined by Miller and co-workers (1998). Strong variations in the genotoxic response to thimerosal were seen, but no evidence of the involvement of the *GSTM1* genotype was observed (Table 1).

Three *GSTM1*-negative and three *GSTM1*-positive individuals of different *GSTT1* and *GSTP1* genotypes were included in the study (Table 2). Negative or weak effects of thimerosal could not be attributed to any *GST* genotype.

Two experiments were regarded as negative: one experiment revealed a non-significant elevation of micronuclei (the second experiment with blood cells of donor 2), the other showed a significant increase only with 0.6 μg/ml thimerosal (the first experiment with blood cells of donor 3). Concentrations at ≥0.6 μg/ml thimerosal were toxic, resulting in a reduced NDI and/or reduced total cell count. Toxicity-related elevation of micronuclei was seen at ≥0.6 μg/ml thimerosal (Table 1).

When thimerosal was prepared one day before use, the aged substance resulted in a variable response (Table 3). Additionally, enhanced Giemsa staining was seen in some experiments with the aged substance, so that samples could frequently not be evaluated under such conditions.

Discussion

Contradictory and inconclusive studies on clastogenic effects of thimerosal in vitro prompted us to reinvestigate thimerosal in the cytochalasin B block micronucleus test with primary human lymphocytes. We found a significant, partly dose-dependent increase of micronuclei at the relatively low concentrations of 0.05–0.5 μg/ml thimerosal; this confirms earlier results obtained with V79 cells (Seelbach et al. 1993) and human lymphocytes (Migliore and Nieri 1991). Since vaccines can contain up to 50 μg/ml (Ball et al. 2001) and 100 μg/ml thimerosal (Fischer et al. 2001), genotoxic effects were seen at concentrations that can be reached at the injection site. Toxicity occurred at ≥0.6 μg/ml thimerosal. A toxicity-related increase of the aberration frequencies was observed at and above this concentration in four experiments.

We observed strong effects at non-toxic concentrations in 14 out of the 16 experiments that could be evaluated. In contrast, earlier studies on micronuclei induction by thimerosal in human lymphocytes showed either weak micronuclei induction in two out of three experiments (Migliore and Nieri 1991) or negative results (van Hummelen and Kirsch-Volders 1992). However, these reports are difficult to evaluate, since the corresponding positive controls were not stated.

We observed pronounced intra- and inter-individual variations in the toxic and genotoxic response to thimerosal. Reproducibility of micronuclei counts can be limited by culture performance and hypotonic treatment. Damaged cells do not seem to sustain the hypotonic treatment as well as non-damaged cells do. The latter may have led to lower micronuclei counts in some of our experiments. Therefore, the concentration range in which the genotoxicity of thimerosal was observed was not identical in each experiment or blood donor. In addition, the concentration–effect curve was very steep. This may have accounted for negative results, since van Hummelen and Kirsch-Volders (1992) did two experiments with one blood donor and observed a reduced cell count at 0.125 μg/ml and toxicity at 0.25 μg/ml. This prompted them to use a lower concentration range (0.06 and 0.12 μg/ml) for the next experiment with another blood donor. Therefore, we recommend repeated, independent tests for the fixation of the concentration range, especially when blood is contributed by different donors.

Contradictory results in studies on the genotoxic effects of thimerosal could be due to differences in the metabolic competence of the cell lines used. Differential lineage expression of *GST* was shown in hematopoietic cell lines. This led to the proposal that GSTT1 and GSTA1 have a greater protective role in the erythroid cells, and GSTM1 in the lymphoid cells (Wang et al. 2000). Cell lines containing high GSTP1 activity can be resistant towards various substrates (Tew 1994). Differences in the susceptibilities of various cells towards toxic or genotoxic stress can additionally be dependent on their origin (organ and donor).

There is evidence that the detoxification of thimerosal or its decomposition products is glutathione-dependent. Thimerosal toxicity, including its sensitizing properties, was attributed to the formation of ethylmercury (Dierickx 1985; Santucci et al. 1998). Ethylmercury can react spontaneously with glutathione and was reported to bind directly to glutathione S-transferases. This was proposed to have a protective effect (Dierickx 1985). Epidemiological evidence for a direct role of GSTM1 in the detoxification of thimerosal or its decomposition products was provided by investigations which showed that the *GSTM1*-negative genotype was significantly more common among thimerosal-sensitized than in non-sensitized individuals (Westphal et al. 2000).

Table 1 Micronuclei (MN) and nuclear division index (NDI) observed in experiments with six blood donors of different glutathione S-transferase statuses. Numbers are given per 1000 cells evaluated unless stated differently. For each blood donor at least two independent experiments were performed

	Concentration										
	Thimerosal									Mitomycin	
	(μg/ml)									(μmol/ml)	
	0.0	0.025	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.08	0.1
Donor 1											
Experiment 1											
MN	5	n.d.	11	8	15*	30***	28**	25/500	10/500	17*	
NDI	1.90	n.d.	1.80	1.70	1.60	1.70	1.60	1.40	1.20	1.70	
Experiment 2											
MN	5	n.d.	3	2	8	22*	10	31***	n.d.		32***
NDI	1.60	n.d.	1.60	1.40	1.60	1.40	1.40	1.40	n.d.		1.40
Donor 2											
Experiment 1											
MN	10	15	22*	14	13	6	16	9	n.d.		29*
NDI	1.36	1.64	1.62	1.75	1.76	1.77	1.48	1.47	n.d.		1.88
Experiment 2											
MN	10	11	10	8	12	15	18	n.e.	n.e.		29*
NDI	1.57	1.50	1.64	1.53	1.37	1.51	1.40	n.e.	n.e.		1.88
Experiment 3											
MN	6	n.d.	2	12	26*	n.e.	60***	n.e.	n.d.		45***
NDI	1.4	n.d.	1.5	1.42	1.36	n.e.	1.33	n.e.	n.d.		1.49
Experiment 4											
MN	4	n.d.	3	9	19*	18*	4/200	n.e.	n.d.		45***
NDI	1.37	n.d.	1.51	1.58	1.31	1.30	1.25	n.e.	n.d.		1.49
Donor 3											
Experiment 1											
MN	14	7	7	7	9	16	7	16	35*		71***
NDI	1.83	1.72	1.72	1.62	1.64	1.56	1.71	1.64	1.34		1.73
Experiment 2											
MN	15	n.d.	10	6	36*	5	7	0	0		41**
NDI	1.97	n.d.	1.91	1.57	1.64	1.50	1.48	1.17	1.17		1.60
Experiment 3											
MN	6	n.d.	5	10	25**	33***	9/500	23/500	38/500		72***
NDI	1.70	n.d.	1.74	1.80	1.70	1.51	1.35	1.22	1.57		1.65
Experiment 4											
MN	12	n.d.	14	16	17	17	31*	n.e.	n.d.		53***
NDI	1.80	n.d.	1.86	1.63	1.60	1.61	1.45	n.e.	n.d.		1.65
Donor 4											
Experiment 1											
MN	2	7	12*	24***	n.e.	n.e.	n.e.	n.e.	n.d.		84***
NDI	1.80	1.60	1.70	1.70	n.e.	n.e.	n.e.	n.e.	n.d.		1.5
Experiment 2											
MN	13	4	12	6	5	38**	n.e.	n.e.	n.d.		102***
NDI	1.44	1.56	1.45	1.35	1.47	1.52	1.20	1.04	n.d.		1.50
Donor 5											
Experiment 1											
MN	9	9	9	16	60***	46***	n.e.	n.e.	n.d.		45***
NDI	1.50	1.36	1.38	1.47	1.45	1.46	1.16	n.e.	n.d.		1.73
Experiment 2											
MN	2	n.d.	n.d.	3	2	2	10*	16**	23***		125***
NDI	1.66	n.d.	n.d.	1.61	1.44	1.74	1.68	1.57	1.52		1.35
Donor 6											
Experiment 1											
MN	5	n.d.	9	5	12	9	8	20*	n.d.		18*
NDI	1.60	n.d.	1.60	1.70	1.70	1.70	1.60	1.50	n.d.		1.70
Experiment 2											
MN	4	n.d.	7	8	9	14*	22**	3/200	n.d.		29***
NDI	1.70	n.d.	1.70	1.80	1.70	1.60	1.60	1.20	n.d.		1.70

n.d., not determined; n.e., could not be evaluated
*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$

Table 2 Genotypes of the blood donors: 1, positive or wild type; 2, variant allele; 0, deficient. *GSTM1* and *GSTT1* were not differentiated into heterozygous or homozygous positive genotypes

	GSTM1	*GSTT1*	*GSTP1*	
			exon 5	exon 6
Donor 1	1	1	11	12
Donor 2	1	1	22	22
Donor 3	1	0	22	12
Donor 4	0	1	12	12
Donor 5	0	1	12	11
Donor 6	0	1	22	22

Table 3 Micronuclei (MN) and nuclear division index (NDI) observed in experiments with freshly prepared and one-day-aged thimerosal. Three independent experiments with different blood donors were performed

Thimerosal concentration (μg/ml)	Freshly prepared		Aged	
	MN	NDI	MN	NDI
Experiment 1				
0	2/1.68			
0.2	2	1.72	2	1.82
0.3	2	1.85	9*	1.69
0.4	6	1.72	6	1.67
0.5	43***	1.58	12*	1.75
Mitomycin (0.1 μmol/ml)	52***/1.57			
Experiment 2				
0	9/1.90			
0.2	9	1.92	13	1.92
0.3	8	1.75	n.e.	n.e.
0.4	24*	1.76	n.e.	n.e.
0.5	8	1.67	21*	1.58
Mitomycin (0.1 μmol/ml)	53*** and 84***/1.50 and 1.58			
Experiment 3				
0	3/1.72			
0.2	2	1.79	3	1.83
0.3	29***	1.73	23***	1.70
0.4	15**	1.63	36***	1.59
0.5	24***	1.37	48***	1.54
Mitomycin (0.1 μmol/ml)	85***/1.52			

n.e., could not be evaluated
*$P < 0.05$; **$P < 0.002$; ***$P < 0.0001$

Therefore, we investigated a possible contribution of *GST* polymorphisms to the detoxification of thimerosal, and focussed on whether certain genotypes could lead to negative results in the micronucleus test with thimerosal. Since there is epidemiological evidence for an involvement of GSTM1 in the detoxification of thimerosal (Westphal et al. 2000), we focussed on GSTM1. *GSTM1* expression in human lymphocytes was shown by RT-PCR (Reich et al. 1999). In fact, there were striking inter-individual differences between the blood donors in the genotoxic response towards thimerosal. However, we did not observe any association between the genotoxic response to thimerosal and any *GST* genotype.

We performed additional experiments to examine a possible influence of thimerosal degradation (Tan and Parkin 2000). For this purpose, thimerosal was dissolved in water and kept for 24 h at 4–8 °C. However, no reproducible trend due to aging of the substance could be found (Table 2). In some experiments with aged thimerosal, enhanced Giemsa staining was seen. This often resulted in samples difficult to evaluate.

Although the results of in vitro assays can raise concern, they have to be seen in the context of other available data, including on the structure/activity relationship, epidemiological data, or animal bioassays. Thimerosal is a common contact sensitiser. High sensitization rates were observed in contact-allergic patients (Aberer and Kraenke 1995) and in health care workers (Schnuch et al. 1998). Electrophilicity and the ability to form adducts with proteins and DNA is a common characteristic of some contact sensitisers and genotoxic carcinogens. Thus, the National Toxicology Program of the USA evaluated 146 chemicals that had been studied for tumorigenicity and mutagenicity. About 20–28% of contact sensitizers were found to be mutagenic and/or tumorigenic (Albert and Magee 2000).

Possible carcinogenic effects of thimerosal were investigated in one study on the chronic toxicity in Fischer 344 rats (Mason et al. 1971). In accordance with the intended use, the substance was applied by subcutaneous injection (0.1, 0.3, and 1.0 mg/kg body weight in 250 μl physiological saline, twice weekly for 12 months). Half of the animals of each experimental group were killed after 12 months and the other animals were kept for an additional 6 months. A non-significant increase of tumours was found in the high-dose thimerosal group. Six other compounds (benzethonium chloride, ethylene chlorohydrin, ethylene glycol, methylparaben, phenol red, and pyridine) were examined in parallel experiments. Benzethonium chloride induced 16 tumours at the injection site in 100 treated male rats and 10 tumours in 100 female rats, whereas no tumours were found in the vehicle-treated group. All other test compounds induced 2 tumours at the injection site in 100 male rats, with the exception of thimerosal, which induced 2 tumours at the injection site in 100 male and female rats each. All the tumours in the thimerosal-treated animals were seen in the high-dose group ($n = 50$). As such, the tumour incidence was not statistically significant. However, it was dose-dependent and the animals were only dosed for 12 months. Therefore, this study does not rule out a possible carcinogenic effect of thimerosal.

In conclusion, thimerosal induced strong effects in the cytochalasin B block in vitro micronucleus test in human lymphocytes. Inter-individual differences in the response were not linked to different *GST* genotypes. Since thimerosal was repeatedly shown to be genotoxic in vitro and in vivo, there is reason for concern about its widespread use.

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