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Age-related alterations in cell division and cell cycle kinetics in control and trimethyltin-treated lymphocytes of human individuals

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Trimethyltin chloride induced age-related suppression of cell division and cell cycle kinetics in human peripheral blood lymphocytes cultured in RPMI 1640 culture medium supplemented with human AB serum, phytohemagglutinin and bromodeoxyuridine. A high frequency of M_1 (first metaphase) cells was seen in cultures treated with a high dose (C_1 = 1.0 μg per culture) and in lymphocytes from donors in the age range 40–70 years. The delay in cell division and cell cycle kinetics may indicate a longer duration in DNA synthesis induced by trimethyltin chloride in aged lymphocytes.

Keywords: aging, cell cycle kinetics, cell division, lymphocytes, trimethyltin chloride

Introduction

As a consequence of the fast developments in industrialization, the introduction of 2000 new man-made chemicals into the atmospheric environment has become an important issue of increasing scientific and public concern. Tin compounds (group IVB) are observed to be an environmental contaminant (Young & Fechter 1986), and are unsurpassed by any other metal in the multiplicity of applications in divergent fields such as stabilizers, catalysts, general and agricultural biocides, antifoulants, preservatives, hospital disinfectants, and dental and veterinary medicines (WHO 1980). As a result, tin compounds are widely dispersed throughout the ecosphere and tin can be accumulated in the food chain via the tin geocycle (Craig 1988). Migration of tin from plastics and tin-plated containers into food and beverages, from plastic medical devices into body fluids or from biocidal compounds are the probable sources by which this metal causes toxicological effects in man and in other organisms.

Age-related alteration of micronucleus formation, chromosomal abnormalities and sister chromatid exchange were recorded in lymphocytes subjected to trimethyltin *in vitro*; however, information available does not include reports on age-related changes in cell division and cell generation cycles. Age had been shown to affect DNA damage in the lymphocyte population of human individuals (Tice & Setlow 1985, Ghosh *et al.* 1990, 1991a, Ganguly 1993).

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Therefore, an *in vitro* study with trimethyltin chloride was carried out in order to determine the cytotoxicity of this chemical by considering mitotic and replicative indices as endpoints, and a final comparison was made in terms of toxicity related to human ageing. The lymphocytic cell generation cycle has recently been proposed as an alternative for assessing mutagenic agents, and monitoring of both control and exposed individuals has provided important guidelines (Rojas *et al.* 1992).

Materials and methods

Trimethyltin chloride (Fluka AG, Buchs, Switzerland) was selected according to Preston *et al.* (1987) following administration of different concentrations to determine the maximum concentration that reduced the replicative index by about 50%. The lower and higher doses used were 0.5 and 1.0 μg per 5 ml culture. Fresh solutions were prepared in sterile distilled water.

Cytogenetic protocol

Human peripheral lymphocytes were collected from healthy male and female voluntary donors, who had not knowingly been exposed to toxic agents. A small sample from children of some voluntary donors was also included. Donors were categorized into seven age groups: I = 1–10 years, II = 11–20 years, III = 21–30 years, IV = 31–40 years, V = 41–50 years, VI = 51–60 years and VII = 61–70 years. Four subjects of both sexes were maintained in each age group. The mean age of the combined population was 35.14 ± 11.42.

To minimize the possible sources of variation, a safe cytogenetic protocol was adopted. Venous blood samples (2 5 ml) were collected by venipuncture, heparinized and 0.3 ml of whole blood was inoculated into RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with heat inactivated human AB serum and phytohemagglutinin (Gibco). Bromodeoxyuridine (Sigma, St Louis, MO, USA) was simultaneously added to each culture at a final concentration of 8 $\mu g\,ml^{-1}$. The target chemical was added at G_0 hour (WHO 1985). Control and treated cultures were maintained in replicate sets giving a total of six cultures for each blood sample. The treatment priod was 72 h at 37°C (Purchase *et al.* 1978, Ghosh *et al.* 1989) in the dark. The medium, serum, phytohemagglutinin and BrdU were of the same lot number and kept frozen. Cultures were fixed after 72 h of incubation at 37°C following a terminal 2 h treatment with colchicine and hypotonic treatment with 0.09% NaCl. Cells were collected onto clean, chilled glass slides and stained following the FPG method.

Data analysis

All cultures were coded prior to data analysis. For each experimental set 1000 cells (both dividing and non-dividing) were scored to compute the mitotic index (MI) and 200 well-differentiated metaphase plates were observed for cell cycle kinetics from the different areas of the slide for each subject. The MI was calculated as (Ghosh 1988)

$$MI = \frac{\text{number of dividing cells}}{\text{total cells observed}} \times 100$$

Table 1. Data on alterations in the rate of cell division in lymphocytes exposed to trimethyltin *in vitro*

Age groups	Doses	Male		Female	
		range	mean ± SD	range	mean ± SD
I	untreated	6.14–8.83	7.26 ± 1.15	7.05–8.27	7.33 ± 0.95
	lower dose	3.80–4.76	4.34 ± 0.40	3.71–6.17	4.89 ± 1.01
	higher dose	2.88–4.00	3.61 ± 0.52	0.70–1.21	1.01 ± 0.22
	trend test *P* value		$P < 0.05$		$P < 0.05$
II	untreated	6.80–7.83	7.11 ± 0.49	6.30–8.09	7.16 ± 0.78
	lower dose	3.85–5.70	4.71 ± 0.77	3.20–7.36	4.64 ± 1.87
	higher dose	2.20–3.02	2.69 ± 0.35	1.08–1.60	1.28 ± 0.23
	trend test *P* value		$P < 0.01$		$P < 0.01$
III	untreated	5.02–7.98	6.48 ± 1.27	6.10–7.61	6.90 ± 0.68
	lower dose	3.90–4.90	4.24 ± 0.45	3.10–4.73	3.81 ± 0.69
	higher dose	1.11–3.75	2.18 ± 1.26	1.19–2.75	1.73 ± 0.72
	trend test *P* value		$P < 0.01$		$P < 0.001$
IV	untreated	5.35 7.30	6.25 ± 0.81	5.58–7.30	6.47 ± 0.85
	lower dose	2.22–4.04	3.25 ± 0.76	3.39–5.00	3.84 ± 0.78
	higher dose	1.20–2.60	2.15 ± 0.65	1.09–1.77	1.36 ± 0.32
	trend test *P* value		$P < 0.01$		$P < 0.001$
V	untreated	3.30 5.82	4.66 ± 1.21	3.25–6.30	4.89 ± 1.33
	lower dose	2.51–3.05	2.83 ± 0.25	1.45–2.71	2.04 ± 0.52
	higher dose	1.25–2.30	1.77 ± 0.57	0.41–0.91	0.75 ± 0.23
	trend test *P* value		$P < 0.01$		$P < 0.001$
VI	untreated	2.80–4.02	3.52 ± 0.52	3.33–4.52	3.93 ± 0.60
	lower dose	1.60–3.49	2.51 ± 0.82	1.28–2.21	1.76 ± 0.47
	higher dose	0.74–1.20	0.96 ± 0.23	0.61–0.81	0.73 ± 0.09
	trend test *P* value		NS		$P < 0.01$
VII	untreated	2.02–4.48	3.24 ± 1.04		
	lower dose	0.73–1.54	1.30 ± 0.52	ND	
	higher dose	0.40–1.05	0.81 ± 0.28		
	trend test *P* value		$P < 0.01$		

ND, not done.

and cell cycle kinetics were analyzed from the replicative index (RI), which was calculated as

$$RI = \frac{1 \times M_1 + 2 \times M_2 + 3 \times M_3}{\text{total metaphases observed}} \times 100$$

by scoring first (M_1), second (M_2) and third (M_3) generation metaphases (Schneider & Lewis 1981). Pooled data were analyzed statistically by the one-tailed trend test and two-way ANOVA (analysis of variance with replication) following Tukey's test (Scheffé 1959).

Results

The mean results were expressed as MI (percentage of cell division) and RI in control and treated lymphocytes from different age groups (Tables 1 and 2). In Tables 1 and 2 the treated male and female lymphocytes showed a 2-fold depression in cell division and cell cycle kinetics compared with controls in almost all age groups, with a higher depression in females. In treated as well as untreated lymphocytes the MI and RI frequencies were lower in the age range from 40 to 70 years in both sexes. From statistical analysis at the group level the mean data revealed a significant positive trend of dose effects for lowering the rate of cell division and cell cycle kinetics in both sexes. The effects of age showed a linear relationship for depression of MI as well as RI (Tables 1 and 2).

The ANOVA test for the mean depression of MI and RI revealed significant variations between subgroups, age groups and chemical concentrations in both sexes (Table 3). Significant interaction of donor's age and the target chemical

Table 2. Data on alterations in the cell cycle kinetics in lymphocytes exposed to trimethyltin *in vitro*

Age groups	Doses	Male		Female	
		range	mean ± SD	range	mean ± SD
I	untreated	2.58–2.81	2.69 ± 0.11	2.66–2.86	2.78 ± 0.09
	lower dose	2.42–2.74	2.58 ± 0.14	2.57–2.83	2.71 ± 0.11
	higher dose	2.27–2.53	2.39 ± 0.11	1.84–2.74	2.28 ± 0.37
	trend test P value		$P < 0.01$		$P < 0.001$
II	untreated	2.55–2.74	2.62 ± 0.08	2.44–2.80	2.66 ± 0.16
	lower dose	2.35 2.65	2.54 ± 0.13	2.20–2.78	2.60 ± 0.27
	higher dose	2.06–2.42	2.30 ± 0.16	1.93–2.29	2.10 ± 0.15
	trend test P value		$P < 0.01$		$P < 0.001$
III	untreated	2.46–2.63	2.54 ± 0.08	2.27–2.86	2.58 ± 0.25
	lower dose	2.42–2.65	2.52 ± 0.10	2.36–2.67	2.51 ± 0.13
	higher dose	1.46–2.70	2.26 ± 0.56	1.99–2.17	2.06 ± 0.08
	trend test P value		$P < 0.01$		$P < 0.001$
IV	untreated	2.25 2.61	2.49 ± 0.16	2.44–2.71	2.60 ± 0.12
	lower dose	2.33–2.58	2.46 ± 0.11	2.35–2.78	2.53 ± 0.21
	higher dose	1.85–2.46	2.15 ± 0.34	1.43–2.53	2.04 ± 0.46
	trend test P value		$P < 0.001$		$P < 0.001$
V	untreated	2.19–2.67	2.45 ± 0.20	2.50–2.65	2.56 ± 0.66
	lower dose	1.90–2.61	2.41 ± 0.34	2.43–2.53	2.47 ± 0.05
	higher dose	1.62–2.63	2.12 ± 0.46	1.83–2.19	2.05 ± 0.15
	trend test P value		$P < 0.001$		$P < 0.001$
VI	untreated	2.33–2.53	2.44 ± 0.09	2.20–2.69	2.50 ± 0.22
	lower dose	2.09–2.44	2.29 ± 0.15	1.99–2.68	2.43 ± 0.31
	higher dose	1.74–2.30	2.02 ± 0.23	1.29 1.86	1.52 ± 0.26
	trend test P value		$P < 0.001$		$P < 0.001$
VII	untreated	2.18–2.79	2.40 ± 0.29		
	lower dose	1.80–2.77	2.34 ± 0.45	ND	
	higher dose	1.68–2.07	1.83 ± 0.17		
	trend test P value		$P < 0.001$		

Table 3. ANOVA of mean mitotic (MI) and replicative indices (RI) in control and treated lymphocytes of different age groups

	Source of variation	d.f.	SS	MS	F	P value
ANOVA for MI in male						
(a)	Between subgroups	20	298.582	14.929	26.036	$P<0.001$
(b)	Between age groups	6	113.962	18.876	32.920	$P<0.001$
(c)	Between concentrations	2	172.962	86.481	150.821	$P<0.001$
(d)	Interaction (b × c)	12	12.359	1.029	1.796	NS
(e)	Between individuals within subgroups	63	36.128	0.573		
ANOVA for MI in female						
(a)	Between subgroups	17	372.255	21.897	33.740	$P<0.001$
(b)	Between age groups	5	57.839	11.567	17.824	$P<0.001$
(c)	Between concentrations	2	296.482	148.241	228.414	$P<0.001$
(d)	Interaction (b × c)	10	17.933	1.793	2.763	$P<0.01$
(e)	Between individuals within subgroups	54	35.047	0.649		
ANOVA for RI in male						
(a)	Between subgroups	20	3.551	0.177	4.775	<0.001
(b)	Between age groups	6	1.216	0.202	5.997	<0.001
(c)	Between concentrations	2	2.156	1.078	31.893	<0.001
(d)	Interaction (b × c)	12	0.188	0.015	0.464	NS
(e)	Between individuals within subgroups	63	2.131	0.033		
ANOVA for RI in female						
(a)	Between subgroups	17	6.977	0.410	8.532	<0.001
(b)	Between age groups	5	1.235	0.247	5.135	<0.001
(c)	Between concentrations	2	5.270	2.635	54.787	<0.001
(d)	Interaction (b × c)	10	0.471	0.047	0.979	NS
(e)	Between individuals within subgroups	54	2.599	0.048		

d.f., degrees of freedom; SS, sum of squared observation; MS, mean squared; NS, not significant.

was noticed only for the MI in females. In Tukey's test the underlined factors are not statistically significant and non-underlined areas indicate significant differences among themselves at the 0.05% level (Table 4). For MI, significant variations among different age groups are of a similar nature in male and female. When the doses were compared, significant differences between control (C_0) and the chemical concentrations (C_1 and C_2) were observed in both sexes, whereas between the two doses the difference was only significant in females (Table 4). In cell cycle kinetics, Tukey's test revealed the similar nature of significant differences between age groups and between doses in both sexes. The comparison of experimental sets revealed that the higher dose (C_2) significantly depressed cell cycle kinetics, but the difference between the two doses was not significant in the two sexes (Table 4).

Discussion

Studies of the genetic hazards due to exposure to organotin compounds *in vitro* and *in vivo* have mainly concentrated on DNA damage. Significant elevation of chromosomal aberrations, sister chromatid exchange, micronucleus counts, and depression of cell division and cell proliferation kinetics in cultured peripheral lymphocytes *in vitro* (Ghosh *et al.* 1989) and *in vivo* in tin miners and patients with lung cancer who had worked in the Yunan tin mines (China) showed the carcinogenic effect of tin (Hu *et al.* 1987). Immunotoxicity and cytotoxicity of trialkyltin compounds had been reported earlier in thymus-dependent cells and cortical lymphocytes (Snoeij *et al.* 1986). *In vivo* genotoxicity in the mouse dominant lethal assay (Epstein *et al.* 1972) and bone marrow chromosomal aberrations (Ganguly 1994) indicate that these compounds are potentially genotoxic. Furthermore, trimethyltin is able to induce chromosomal supercontraction, indicating its spindle-inhibiting potency (Jensen *et al.* 1991). This is further supported by the observation of a high frequency of aneuploidy, *c* mitosis and endoreduplication (Ghosh 1988, Ghosh *et al.* 1989).

Age has been shown to significantly affect the frequency of spontaneous micronucleus formation or DNA damage in lymphocyte populations of male and female individuals (Tice & Setlow 1985, Ghosh *et al.* 1990, Ganguly 1993). One of the predictions of theories of aging involving DNA integrity is that cells from older members of a species should exhibit increased levels of damaged DNA, possibly accompanied by a decreased efficiency in the recognition and repair of induced damage (Tice & Setlow 1985, Singh *et al.* 1990). Age-related induction of chromosomal aberrations and micronucleus

Table 4. Turkey's test for mean mitotic (MI) and replicative indices (RI) in control and treated sets of different age groups

Tukey's test for MI (male)								
(a)	Mean values in different age groups arranged in increasing order							
	Age groups	VII	VI	V	IV	III	II	I
	Sample means	1.78	2.32	3.08	3.88	4.29	4.83	5.06
(b)	Mean values in different concentrations arranged in increasing order							
	Chemical concentrations			C_2	C_1	C_0		
	Sample means			2.02	3.30	4.42		
Tukey's test for MI (female)								
(a)	Mean values in different age groups arranged in increasing order							
	Age groups		VI	V	IV	III	II	I
	Sample means		2.14	2.56	3.88	4.14	4.36	4.40
(b)	Mean values in different concentrations arranged in increasing order							
	Concentrations			C_2	C_1	C_0		
	Sample means			1.14	3.49	6.11		
Tukey's test for RI (male)								
(a)	Mean values in different age groups arranged in increasing order							
	Age groups	VII	VI	V	IV	III	II	I
	Sample means	2.18	2.24	2.32	2.36	2.43	2.48	2.55
(b)	Mean values in different concentrations arranged in increasing order							
	Chemical concentrations			C_2	C_1	C_0		
	Sample means			2.14	2.44	2.51		
Tukey's test for RI (female)								
(a)	Mean values in different age groups arranged in increasing order							
	Age groups		VI	V	IV	III	II	I
	Sample means		2.15	2.36	2.38	2.39	2.45	2.59
(b)	Mean values in different concentrations arranged in increasing order							
	Concentrations			C_2	C_1	C_0		
	Sample means			2.01	2.54	2.61		

C_2 (1.0 μg), C_1 (0.5 μg) and C_0 (control). Underlined factors are not significantly different at the 0.05% level.

formation in peripheral lymphocytes exposed to trimethyltin has been reported by Ghosh *et al.* (1990, 1991a). However, little attention has been paid to cell proliferation kinetics and cell division in the presence of trimethyltin in lymphocytes of different age groups. In the present report, cell division and cell cycle kinetics are considered as two major endpoints. A sharp linear relationship between donor age and spontaneous depression of cell division and cell generation cycles were noticed in both sexes. Donors aged 40–70 years were more vulnerable for these endpoints in both sexes. Trimethyltin induced a significant level of suppression of cell division and cell cycle kinetics *in vitro*, which is further supported by Ghosh *et al.* (1989). In the present context trimethyltin induced age-related depression with much higher frequencies of first cycle metaphase (M_1) cells, and bears a fine connection with the age-related induction of chromosomal aberrations and micronuclei *in vitro* (Ghosh *et al.* 1990, 1991a).

Division delay has been proposed to be associated with a longer duration of DNA duplication (Chaganti *et al.* 1974)

and earlier reports suggested that a delay in cell cycle kinetics seems to be responsible for DNA damage. In this regard, aging would be defined as a direct consequence of detrimental accumulation of unrepaired DNA lesions and, as the proportion of such cells increases in various tissues, the organism ages. In this event, unrepaired DNA lesions should increase at substantially faster rates as the level of repair ability is reduced with advancing age. Earlier investigators concluded that exposure to chemicals or ionizing radiation did accelerate the normal rate of aging. Thus the age-related induction of division delay and delay in cell cycle kinetics by trimethyltin is justified by the delay in DNA synthesis and this is further supported by the evidence that the organotin compounds are potent inhibitors of DNA, RNA and protein synthesis in experimental animals (Snoeij *et al.* 1986). The suppression of cell proliferation by organotin compounds is further supported by Röderer (1982) with an observation of disturbances in mitosis and cytokinesis in algae. In accordance with some of these findings, trimethyltin compounds have been found to inhibit *in vitro* polymerization of rat brain tubulin (Tan *et al.* 1978).

Cell division and cell cycle kinetics are affected by several factors such as donor age, health factors and culture conditions (Ghosh *et al.* 1991b). Moreover, interindividual variations are considered to be important in screening the effects of chemicals, and are related to a variety of factors such as age, metabolism and intrinsic repair competence (Paigen *et al.* 1991), as well as to the different susceptibility of lymphocyte subpopulations (Beek & Obe, 1974). In the present experiments, interindividual variability has frequently appeared in both untreated and treated lymphocytes of both sexes.

The level of toxicity of the organotin compounds appears to be determined by the number and nature of organic groups and by the increase in the chain length of alkyl groups. Triorganotins act as effective inhibitors of mitochondrial ATP synthesis (Penninks *et al.* 1983) and trimethyltin is a potent inhibitor of oxidative phosphorylation in mitrochondria (Aldridge & Street 1971). Thus damage in trimethyltin-treated cells would persist and would not be easily repaired. Rose (1969) identified that one molecule of hemoglobin binds two molecules of trialkyltin. In another report, George & Cramp (1989) mentioned that inhibition of ATP synthesis could produce a delay in the repair of chromosomal damage. A similar phenomenon could explain the action of trimethyltin. Thus, to validate a new biological system, cell division and cell cycle kinetics could be considered as reproducible endpoints in genetic toxicity testing.

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