J Occup Health 2009; 51: 498–512

Journal of Occupational Health

Genotoxicity Studies of Heavy Metals: Lead, Bismuth, Indium, Silver and Antimony

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Abstract: Genotoxicity Studies of Heavy Metals: Lead, Bismuth, Indium, Silver and Antimony: Keiko ASAKURA, *et al*. Department of Preventive Medicine and Public Health, Keio University School of Medicine—Objectives: Many kinds of heavy metals are used in industry; thus, it is important for us to clarify their toxicity. For example, lead, which is a component of solder, is notorious for its neurotoxicity, and substitute materials have been sought for many years. Therefore, we examined the genotoxicity of lead and also those of metallic bismuth, indium, silver and antimony which are possible substitutes for lead in solder. **Methods:** Bacterial reverse mutation tests and chromosomal aberration tests in cultured mammalian cells were performed according to standard procedures. **Results:** Antimony showed genotoxicity in both tests, and bismuth also showed positive results in the chromosomal aberration test. In contrast, lead, indium, and silver were considered to be inactive by the criteria of the present study. **Conclusions:** Although further studies are needed because of the difficulty of genotoxicity evaluation using an *in vitro* system, sufficient precautions should be made when antimony and bismuth are used.
(J Occup Health 2009; 51: 498–512)

Key words: Bacterial reverse mutation test, Chromosomal aberration test in cultured mammalian cells, Genotoxicity, Heavy metals

Lead-containing solder has been widely used as a basic joint material in the electrical and electronic industries. However, lead has apparent neurotoxicity and can also damage the hematopoietic, renal, and skeletal systems[1)]. In addition, it is known that children are the most vulnerable population to the neurotoxicity of lead. To minimize lead accumulation in the environment, lead-free solder is now being introduced into electronic industries. However, there is very little information about the genotoxicity of alternative metals used in lead-free solder, such as bismuth, indium, silver, and antimony. Thus, we conducted bacterial mutation tests and chromosomal aberration tests in cultured mammalian cells for these metals as well as lead for a positive control metal. In our previous studies, animal experiments using rats were performed to examine the oral toxicity of bismuth and indium[2, 3)]. These two metals did not show toxicity in both acute oral toxicity studies and 28-day repeated oral dose toxicity studies. Since substances without oral toxicity could still have genotoxicity at the same concentration level, the information provided in this study should help to address the appropriate use of heavy metals.

Received Jun 5, 2009; Accepted Aug 25, 2009
Published online in J-STAGE Oct 23, 2009
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Materials and Methods

Test substance

Five test substances, lead, bismuth, indium, silver and antimony were examined. Lead (abbreviation: Pb, purity: 99.9%, average particle size: 10 μm, lot number: 67244G), bismuth (Bi, 99.9%, 10 μm, 67243G), indium (In, 99%, 45 μm, 67246G), silver (Ag, 99.9%, 10–20 μm, 67245G), and antimony (Sb, 99%, 10 μm, 88711G) were supplied by Kojyundo Chemical Lab. Co., Ltd., and kept at room temperature in a dark place until use.

Bacterial reverse mutation test

The bacterial strains tested in this study were

Table 1. Materials

a) Genetic properties of tested bacterial strains

Strain	Gene affected	Additional mutations			Mutation type
		DNA repair	LPS	R-factor	
TA100	*hisG*	*uvrB*	*rfa*	pKM101	base pair change
TA1535	*hisG*	*uvrB*	*rfa*	–	base pair change
WP2*uvrA*/pKM101	*trpE*	*uvrA*	+	pKM101	base pair change
TA98	*hisD*	*uvrB*	*rfa*	pKM101	frameshift
TA1537	*hisC*	*uvrB*	*rfa*	–	frameshift

b) S9 mix composition (per 1 m*l*)

Components	Bacterial reverse mutation test	Chromosomal aberration test
S9	0.1 m*l*	0.3 m*l*
MgCl2 · 6H20	8 μmol	5 μmol
KCl	33 μmol	33 μmol
D-glucose 6-phosphate	5 μmol	5 μmol
β-NADPH	4 μmol	–
β-NADH	4 μmol	–
β-NADP$^+$	–	4 μmol
Sodium phosphate buffer (pH 7.4)	100 μmol	–
HEPES (pH 7.2)	–	4 μmol
Sterilized purified water	remainder	remainder

c) Control substances

	Substance	Abbreviation	Supplier	Lot no.	Purity (%)
Negative control	Dimethylsulfoxide	DMSO	Wako Pure Chemical Industries, Ltd.	ELH6906	99.5
			or Kanto Chemical Co., Inc.	210G1441	>99.7
	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide	AF-2	Wako Pure Chemical Industries, Ltd.	PAE1151	98.0–102.0
				CAP0185	98.9
Positive control	Sodium azide	NaN_3	Wako Pure Chemical Industries, Ltd.	KSQ2529	98.0
				KWE6685	96.5
	N-ethyl-N'-nitro-N-nitrosoguanidine	ENNG	Sigma Chemical Company	56F-3651	99.0
	9-aminoacridine hydrochloride	9-AA	Aldrich Chemical Company, Inc	TR16323JR	98.0
			or Sigma Chemical Company	80F-0186	>99.0
	2-aminoanthracene	2-AA	Wako Pure Chemical Industries, Ltd.	ACE1396	96.4
				TWH2355	98.0

Salmonella typhimurium TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr*A/pKM101, kindly supplied by Professor B.N. Ames of California University and the Japan Bioassay Research Center. These strains are widely used in bacterial reverse mutation tests and are recommended in the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline[4–6]. Their genetic properties are summarized in Table 1-a).

The stock suspensions of the tester strains were prepared by mixing 4 m*l* of the fresh bacterial suspension, which were then stored below –80°C. A frozen stock culture (20 μ*l*) of each tester strain was inoculated into 10 m*l* of the liquid growth medium and was grown with agitation for 8 hr at 37°C. The bacterial density of the suspension was measured with a turbidimeter, and the cell number of the suspension was calculated from the density.

CLIMEDIA AM-N MEDIUM (purchased from Oriental Yeast Co., Ltd., Lot No. ANI620JP or ANI380FQ) was used as the minimal glucose agar plate.

Table 2. Test conditions for antimony

Tester strain	Concentration (μg/plate)		
	Main tests		Confirmation test
	S9 mix (–)	S9 mix (+)	S9 mix (–)
TA100	5,000, 2,500, 1,250, 625, 313, 156	2,500, 1,250, 625, 313, 156, 78.1, 39.1	–
TA1535	1,250, 625, 313, 156, 78.1, 39.1	2,500, 1,250, 625, 313, 156, 78.1, 39.1	–
WP2*uvrA*/pKM101	5,000, 2,500, 1,250, 625, 313	5,000, 2,500, 1,250, 625, 313	–
TA98	1,250, 625, 313, 156, 78.1, 39.1	5,000, 2,500, 1,250, 625, 313, 156	–
TA1537	5,000, 2,500, 1,250, 625, 313, 156	5,000, 2,500, 1,250, 625, 313, 156	2,250, 2,000, 1,750, 1,500, 1,250, 1,000, 750, 500

Table 3. Positive control for bacterial reverse mutation test

Strain	Without S9 mix (μg/plate)		With S9 mix (μg/plate)		Volume (m*l*/plate)
TA100	AF-2	0.01	2-AA	1	0.1
TA1535	NaN_3	0.5	2-AA	2	0.1
WP2*uvrA*/pKM101	AF-2	0.005	2-AA	2	0.1
	ENNG*	2	2-AA	2	0.1
TA98	AF-2	0.1	2-AA	0.5	0.1
TA1537	9-AA	80	2-AA	2	0.1

*ENNG was used only in the tests for Sb.

Top agar was prepared as a solution of 0.6 g of Bacto-agar (Difco Laboratories, Lot No. 133577JD or 136958JC) and 0.5 g of sodium chloride in 100 m*l* of purified water. An amino acid solution containing 0.5 mM D-biotin and 0.5 mM L-histidine for *Salmonella typhimurium* or 0.5 mM L-tryptophan for *Escherichia coli* was added to this agar solution at a ratio of 1:10.

The S9 mix was purchased from Kikkoman Corporation (Lot No. RAA-436, RAA-432, or RAA-450) and stored below –80°C until use. S9 was prepared from the liver of male Sprague-Dawley rats. Table 1-b) shows the composition per milliliter of S9.

The test method was as follows. The test substances were suspended in DMSO at 50 mg/m*l*, then serially diluted with the same solvent to each test substance concentration. At the same time, negative (solvent) and positive controls were prepared as described in Table 1-c). Sodium azide was dissolved in DW and the other positive controls were dissolved in DMSO.

A dose-finding test was conducted at eight concentrations of 5000, 1250, 313, 78.1, 19.5, 4.88, 1.22, and 0.305 (except Sb) μg/plate. With respect to Pb, Bi, In, and Ag, the test showed that the number of revertant colonies was less than twice that of the corresponding negative (solvent) control in all tester strains with or without S9 mix. Microbial toxicity was not observed in all tester strains with or without S9 mix. Accordingly, the main test was conducted at the following concentrations: 5,000, 2,500, 1,250, 625, and 313 μg/plate both with and without S9 mix. Conversely, in the case of Sb, an increase in the number of revertant colonies was observed in TA1537 without S9 mix, and microbial toxicity was observed in several strains. Thus, the main tests were conducted twice at the concentrations shown in Table 2. In addition, a confirmation study was conducted for Sb due to the discordant results of the main tests.

The main tests were conducted by the preincubation method. For each concentration of assay without metabolic activation, 0.1 m*l* of one of the test substance suspension or the negative control, 0.5 m*l* of 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 m*l* of each bacterial suspension were mixed. For assays with metabolic activation, 0.5 m*l* of S9 mix was added instead of 0.1 M sodium phosphate buffer. After preincubation for 20 min at 37°C, 2 m*l* of the molten top agar was added to this mixture which was then poured onto a minimal glucose agar plate. Lastly, after the agar overlay solidified, the plates were incubated for 48 h at 37°C.

The background lawn was checked by a stereoscopic microscope to examine the level of microbial toxicity. The precipitates in agar plates were also observed by the naked eye. The number of revertant colonies in each plate was counted by an automatic colony counter or by manual counting. Three plates were used for each concentration, together with positive and negative

controls. Positive controls assayed together by the same method are listed in Table 3. As a bacterial contamination check, the highest concentration of test solution or S9 mix was mixed with the top agar and poured over the agar plate after incubation.

The test substance was judged to be mutagenic (or positive) when the mean number of revertant colonies dose-dependently increased two-fold or more than that of the corresponding negative control for at least one tester strain with or without S9 mix. In addition, reproducibility was determined by using results of both the dose-finding tests and main tests. For the test substances that were judged to be positive, specific activities (mean number of revertant colonies per milligram of the test substance) were calculated. The data was not analyzed statistically.

Chromosomal aberration test in cultured mammalian cells

Test substances were suspended in 1% sodium carboxymethylcellulose solution (1% CMC-Na solution, Nacalai Tesque, Inc., Lot no. M0B1469). CMC-Na was also used as a negative control. Mitomycin C (MMC: Kyowa Hakko Kogyo Co., Ltd., lot no. 307AJC, contents 99% or lot no. 317AJD, contents 100% or lot no. 337AJG, contents 103%) and Benzo [a] pyrene (BP; Tokyo Kasei Kogyo Co., Ltd., lot no. GG01, contents 95.6%) were used as positive controls.

The CHL/IU cell line was purchased from Dainippon Pharmaceutical Co., Ltd. S9 was purchased from Kikkoman Corporation (Lot No. RAA-430, RAA-433, or RAA-445).

The test procedure includes two parts: the cell growth inhibition test and the chromosomal aberration test.

1) Cell growth inhibition test

According to the results of preliminary tests, concentrations selected for each test substance were as follows:

Pb) –S9 mix: 62.5, 125, 250, 500, and 1,000 μg/m*l*; +S9 mix: 62.5, 125, 250, 500, and 1,000 μg/m*l*,

Bi) –S9 mix: 78.1, 156, 313, 625, 1,250, 2,500, and 5,000 μg/m*l*; +S9 mix: 313, 625, 1,250, 2,500, and 5,000 μg/m*l*,

In) –S9 mix: 39.1, 78.1, 156, 313, 625, 1,250, 2,500, and 5,000 μg/m*l*; +S9 mix: 156, 313, 625, 1,250, 2,500, and 5,000 μg/m*l*,

Ag) –S9 mix: 313, 625, 1,250, 2,500, and 5,000 μg/m*l*; +S9 mix: 313, 625, 1,250, 2,500, and 5,000 μg/m*l*,

Sb) –S9 mix: 10, 20, 50, 125, 250, and 500 μg/m*l*; +S9 mix: 5, 10, 20, 30, 40, and 50 μg/m*l*.

For this test, 5 m*l* of cell suspensions (4×10^3 cells/m*l*) were incubated for three days in a 6 cm plastic plate, using two plates for each concentration. After removal of the culture medium, the cells were treated with 0.3 m*l* of either test substance suspension or negative control and 2.7 m*l* (for –S9 mix) or 2.2 m*l* (for +S9 mix) of culture medium. Additionally, 0.5 m*l* of S9 mix was added to +S9 mix plates. The cells were treated for 6 h, washed three times, and then incubated in 5 m*l* of fresh growth medium for a further 18 h. After being washed, survival cells were counted with a hemocytometer. The ratio of surviving cells at each concentration to that of the corresponding negative control (cell growth index) was calculated, and a survival curve was drawn to calculate the 50% inhibition concentration of cell growth (IC_{50}).

2) Chromosomal aberration test

2.1) Pulse treatment (main test)

Based on the result of the cell growth inhibition test, the concentrations selected for pulse treatment were: Pb) –S9 mix: 250, 500, and 1,000 μg/m*l*, +S9 mix: 250, 500, and 1,000 μg/m*l*; Bi) –S9 mix: 625, 1,250, 2,500, and 5,000 μg/m*l*, +S9 mix: 1,250, 2,500, and 5,000 μg/m*l*; In) –S9 mix: 156, 313, 625, and 1,250 μg/m*l*, +S9 mix: 625, 1,250, 2,500, and 5,000 μg/m*l*; Ag) –S9 mix: 1,250, 2,500, and 5,000 μg/m*l*, +S9 mix: 1,250, 2,500, and 5,000 μg/m*l*; and Sb) –S9 mix: 12.5, 25, 50, 100, and 200 μg/m*l*, +S9 mix: 6.25, 12.5 25, 50, 100, and 200 μg/m*l*. In the positive controls, the final concentrations of MMC and BP were set at 0.1 and 20 μg/m*l*, respectively.

The cultured cells were treated with the test substance by the same procedure described for the cell growth inhibition test. Cells in positive control groups were treated as described below: –S9 mix: 0.3 m*l* of MMC solution and 2.7 m*l* of culture medium; +S9 mix: 0.015 m*l* of BP solution, 0.5 m*l* of S9 mix, and 2.5 m*l* of culture medium.

Two hours before the end of the cell treatment, colcemid was added to the medium in each plate to a final concentration of 0.1 μg/m*l* in order to accumulate cells in the metaphase. After treatment, the cells were washed with Ca^{2+}, Mg^{2+}–free Dulbecco's phosphate buffer saline (PBS(–); Dulbecco's PBS "Nissui", Nissui Pharmaceutical Co., Ltd.), dissociated with 0.25% trypsin solution, then centrifuged. After removal of the supernatant, 4 m*l* of 0.075 mo*l*/*l* potassium chloride solution was added for hypotonic treatment (37°C, 15 min). The cells were then fixed with 0.5 m*l* of a cold methanol and acetic acid mixture (3:1 v/v). After centrifugation, the supernatant was removed and 4 m*l* of fresh fixative was added. This fixation procedure was repeated two times. Thereafter, the cells were suspended in a small amount of the fixative, and a drop of the cell suspension was placed at two points on a glass slide, then left to dry. The cells were stained using 3% Giemsa's solution. Two slides were prepared for each plate.

Concurrently, the rate of surviving cells at the time of specimen preparation was also measured. A small amount of the cell suspension (for the specimen preparation) was collected and the cell number was counted with a hemocytometer.

The specimens that had 50 or more mitotic cells per plate were used for the survey and the prepared slides were observed as follows. First, the incidence of structural aberration was confirmed to be appropriate in negative and positive controls. Then, the main observation was done using the blind method. One hundred metaphase cells per plate (i.e. 200 cells for each concentration), that had well-spread chromosomes, were observed.

Structural aberrations were classified into 1) chromatid breaks (ctb), 2) chromatid exchanges (cte), 3) chromosome breaks (csb), 4) chromosome exchanges (cse: dicentric, ring, etc.), and 5) fragmentation (frg)[7]. Cells without structural aberration and not having 25 ± 2 chromosomes were omitted from the survey. A "gap" is an achromatic region in a single chromatid that was narrower than the width of the chromatid, but distinguished from the other aberrations and not included in "structural aberrations". Polyploid cells, including endoreduplicated cells, were scored as numerical aberrations.

A cell having at least one structural chromosomal aberration was classified as an aberrant cell. The test substances were judged to have the potential to induce chromosomal aberration (positive: +) if either, or both, of the aberration incidences of two types (structural or numerical) among observed cells was 10% or more. If either or both of the two incidences were 5% or more and less than 10%, the potential was judged inconclusive (±). When both of the incidences were less than 5%, it was judged to be negative (–).

2)-2 Pulse treatment (confirmation test)

When the results were inconclusive, confirmation tests were conducted. The cells were treated with the test substance using the same procedure as in the main test. The test substances were regarded as positive if the reproducibility was confirmed in this study.

2)-3 Continuous treatment

When the results of the pulse treatment were negative, 24 h continuous tests were conducted. The cells were treated with the test substance using the same procedure as in the pulse treatment except that the concentration of the substances and treatment duration (24 h) were different. In the case of Pb, concentrations selected for continuous cell growth inhibition tests were 62.5, 125, 250, 500, and 1,000 μg/m*l*. Similarly, 50, 100, 200, 300, 400, and 500 μg/m*l* were selected for In, and 313, 625, 1,250, 2,500, and 5,000 μg/m*l* for Ag. Based on the test results, 250, 500, and 1,000 μg/m*l* were selected for the chromosomal aberration tests for Pb, 125, 250, 500, and 1,000 μg/m*l* for In, and 1,250, 2,500, and 5,000 μg/m*l* for Ag. An MMC of 0.03 μg/m*l* was used as a positive control.

Results

Bacterial reverse mutation test

1) Lead, Bismuth, Indium, and Silver

The results of the dose-finding test showed that the numbers of revertant colonies were less than twice that of the corresponding negative (solvent) control in all tester strains with or without S9 mix. Microbial toxicity was not observed in any of tester strains with or without S9 mix.

According to these results, the main test was conducted at five concentrations ranging from 5,000 to 313 μg/plate at a common ratio of 2 for all tester strains. In the main test, the numbers of revertant colonies were less than twice that of the corresponding negative (solvent) control in all tester strains with or without S9 mix. Microbial toxicity was not observed in any of tester strains with or without S9 mix. Precipitates were observed at doses of 313 μg/plate or more for bismuth, and at doses of 2,500 μg/plate or more for silver with and without S9 mix.

2) Antimony

In the dose-finding test, an increase in the number of revertant colonies was observed in TA1537 without S9 mix. Also, microbial toxicity was observed in TA100 (on 5,000 μg/plate without S9 mix and 1,250 μg/plate with S9mix), TA1537 (on 5,000 μg/plate with and without S9 mix), TA1535 (on 1,250 μg/plate with and without S9 mix), and TA98 (on 1,250 μg/plate without S9 mix and 5,000 μg/plate with S9 mix). Precipitates were not observed in any plate.

In the main test, the number of revertant colonies was less than twice that of the corresponding negative (solvent) control in all tester strains with or without S9 mix (Table 4-a)). Since this result was incompatible with the result of the dose-finding test, the main test was repeated under the same conditions. In the second test, the number of revertant colonies of TA1537 without S9 mix increased to more than twice that of the negative control (Table 4-b)). Due to the discordance of the two main results, a confirmation test was performed. Only TA1537 was examined in this last test, and the result was the same as the second main test (Table 4-c)). Microbial toxicity was observed in TA100, TA1537, TA1535 and TA98 at high concentration ranges in both the main and confirmation tests.

The negative and positive control values in the tests were within the proper ranges calculated based on historical data. Additionally, the number of revertant colonies induced by the positive controls was more than twice that of the negative controls in all the tester strains both with and without S9 mix. Also, bacterial or fungal contamination, which would have affected the acceptance of the test system, was not observed.

Chromosomal aberration test in cultured mammalian cells

IC_{50} in the cell growth inhibition test for the pulse treatment is shown in Table 5. According to these results,

Table 4. Colony number in bacterial reverse mutation test for antimony

a) Main test 1

Metabolic activation system	Test substance concentration (μg/plate)	Number of revertants (No. of colonies/plate)				
		Base-pair change type			Frameshift type	
		TA100	TA1535	WP*2uvrA*/ pKM101	TA98	TA1537
S9 mix (–)	Negative control	106	8	78	18	13
	39.1	–	8	–	19	–
	78.1	–	9	–	20	–
	156	106	7	–	19	13
	313	99	8	75	15	13
	625	96	9*	72	19	14
	1,250	98	8*	76	15*	24
	2,500	78*	–	77	–	12*
	5,000	0*	–	67	–	0*
	Positive control (name (concentration))	619 (AF-2 (0.01))	450 (NaN_3 (0.5))	3,572 (ENNG (2))	728 (AF-2 (0.1))	216 (9-AA (80))
S9 mix (+)	Negative control	107	10	92	28	18
	39.1	106	7	–	–	–
	78.1	114	11	–	–	–
	156	106	11	–	28	18
	313	98	9	96	29	19
	625	93	9	84	23	16
	1,250	85*	11*	108	26	18
	2,500	74*	7*	96	24	10*
	5,000	–	–	92	0*	0*
	Positive control (name (concentration))	1,275 (2-AA (1.0))	177 (2–AA (2.0))	621 (2–AA (2.0))	308 (2-AA (0.5))	210 (2-AA (2.0))

b) Main test 2

Metabolic activation system	Test substance concentration (μg/plate)	Number of revertants (No. of colonies/plate)				
		Base-pair change type			Frameshift type	
		TA100	TA1535	WP*2uvrA*/ pKM101	TA98	TA1537
S9 mix (–)	Negative control	98	16	67	14	13
	39.1	–	17	–	19	–
	78.1	–	18	–	18	–
	156	111	14	–	21	13
	313	100	20	69	22	10
	625	103	14*	67	15*	16
	1,250	102	16*	69	10*	29
	2,500	57*	–	65	–	9*
	5,000	0*	–	62	–	0*
	Positive control (name (concentration))	539 (AF-2 (0.01))	461 (NaN_3 (0.5))	3,659 (ENNG (2))	783 (AF-2 (0.1))	242 (9-AA (80))
S9 mix (+)	Negative control	98	17	88	25	23
	39.1	96	20	–	–	–
	78.1	99	19	–	–	–
	156	102	19	–	23	18
	313	93	21	98	27	19
	625	94	13	91	31	21
	1,250	74*	10*	94	22	17
	2,500	73*	15*	82	11*	16*
	5,000	–	–	90	0*	0*
	Positive control (name (concentration))	1,378 (2-AA (1.0))	197 (2-AA (2.0))	650 (2-AA (2.0))	370 (2-AA (0.5))	183 (2-AA (2.0))

Table 4. Colony number in bacterial reverse mutation test for antimony (continued)

c) Confirmation test

Metabolic activation system	Test substance concentration (μg/plate)	Number of revertants (No. of colonies/plate)				
		Base-pair change type			Frameshift type	
		TA100	TA1535	WP2*uvrA*/ pKM101	TA98	TA1537
S9 mix (–)	Negative control	–	–	–	–	12
	500	–	–	–	–	22
	750	–	–	–	–	25
	1,000	–	–	–	–	24
	1,250	–	–	–	–	21
	1,500	–	–	–	–	12*
	1,750	–	–	–	–	10*
	2,000	–	–	–	–	7*
	2,250	–	–	–	–	6*
	Positive control (name (concentration))	– (AF-2 (0.01))	– (NaN_3 (0.5))	– (ENNG (2))	– (AF-2 (0.1))	216 (9-AA (80))

The test was performed using three plates for each condition. The numbers indicated in the table are the average colony numbers for three plates. *Microbial toxicity was observed.

Table 5. IC50 in the cell growth inhibition test for the pulse treatment

Test substance	IC50 (μg/m*l*)	
	–S9mix	+S9mix
Bismuth	4,349	>5,000
Lead	>1,000	>1,000
Indium	906	4,791
Silver	>5,000	>5,000
Antimony	60	50

several appropriate concentrations were selected for the main tests. In preliminary observation for the main test, there were 50 or more mitotic cells per plate in all specimens treated with Pb, Bi, and Ag. Therefore, all plates were used for the survey. However, there were less than 50 mitotic cells per plate in several groups treated with In and Sb of high concentration. The specimens which could not be analyzed are noted in Table 6.

In the main test for Pb, the incidences of cells with structurally aberrant chromosomes were 7.0% and 4.0% at 500 and 1,000 μg/m*l*, respectively, in +S9 mix. The incidence of cells with numerically aberrant chromosomes was less than 5% at each concentration in +S9 mix (Table 6-a-1)). For Bi, the incidences of cells with structurally aberrant chromosomes were 5.0% and 6.5% at 2,500 and 5,000 μg/m*l*, respectively, in +S9 mix. The incidence of cells with numerically aberrant chromosomes was less than 5% at each concentration in +S9 mix (Table 6-b-1)). Also, for Sb, the incidences of cells with structurally and numerically aberrant chromosomes were more than 10% under several conditions (Table 6-e)). Since Sb was judged to be positive in the main test, follow-up tests were not performed. By contrast, in the main tests of In and Ag, the incidence of cells with structurally and numerically aberrant chromosomes was less than 5%.

Based on these results, confirmation tests were conducted for Pb and Bi at appropriate concentrations as shown in Table 6. For Pb, the incidences of cells with structurally and numerically aberrant chromosomes were less than 5% in each treatment, and the reproducibility of the result was not confirmed (Table 6-a-2)). Therefore, the judgement was negative for the pulse treatment. In the case of Bi, positive results were observed again; therefore, Bi was judged to have the potential to induce chromosomal aberration, and the following continuous treatment was not conducted.

According to the results of pulse treatment, cell growth inhibition tests using continuous treatment were conducted for Pb, In, and Ag. The incidence of cells with structurally and numerically aberrant chromosomes was less than 5% in each treatment (Table 6). Therefore, the judgement was negative for Pb, In, and Ag.

In both the main and confirmation tests, the incidence of cells with structurally and numerically aberrant chromosomes was less than 5% in the negative control, and more than 10% in the positive control. These results demonstrate that the procedures used in this study were technically appropriate.

Discussion

In the present study, antimony showed positive results (genotoxicity) in both the bacterial reverse mutation test and the chromosomal aberration test in cultured

Table 6. Aberrant cell number in chromosomal aberration test

a-1) Lead, pulse treatment (main test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	3	0	0	0	0	3	0	108	0	0	0
–	control	100	1	0	0	0	0	1	0	92	0	0	0
	(CMC-Na)	200: total	4 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)	0	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	0	0	1	1	78	0	0	0
–	250P	100	2	0	0	0	0	2	0	82	0	0	0
		200: total	3 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	1	80	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	0	0	0	0	0	1	81	0	0	0
–	500P	100	0	0	0	1	0	1	0	67	0	0	0
		200: total	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	1	74	0 (0.0)	0 (0.0)	0 (0.0)
		100	3	0	1	0	0	4	0	78	0	0	0
–	1,000P	100	0	0	0	0	0	0	0	71	0	0	0
		200: total	3 (1.5)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	4 (2.0)	0	74	0 (0.0)	0 (0.0)	0 (0.0)
	Positive	100	58	50	1	0	0	82	4	77	0	0	0
–	control	100	53	48	1	0	0	77	7	66	0	0	0
	(MMC 0.1)	200: total	111 (55.5)	98 (49.0)	2 (1.0)	0 (0.0)	0 (0.0)	159 (79.5)	11	72	0 (0.0)	0 (0.0)	0 (0.0)
	Negative	100	1	0	1	1	0	3	0	106	1	0	1
+	control	100	2	1	0	0	0	3	0	94	0	0	0
	(CMC-Na)	200: total	3 (1.5)	1 (0.5)	1 (0.5)	1 (0.5)	0 (0.0)	6 (3.0)	0	100	1 (0.5)	0 (0.0)	1 (0.5)
		100	1	0	0	0	0	1	0	82	0	0	0
+	250P	100	0	0	0	0	0	0	0	75	0	0	0
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	79	0 (0.0)	0 (0.0)	0 (0.0)
		100	2	1	2	0	0	4	7	75	0	0	1
+	500P	100	9	1	0	0	0	10	1	80	0	0	0
		200: total	11 (5.5)	2 (1.0)	2 (1.0)	0 (0.0)	0 (0.0)	14 (7.0)	8	78	0 (0.0)	0 (0.0)	1 (0.5)
		100	2	0	1	0	0	3	0	79	0	0	0
+	1,000P	100	5	0	0	0	0	5	2	88	2	1	3
		200: total	7 (3.5)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	8 (4.0)	2	83	2 (1.0)	1 (0.5)	3 (1.5)
	Positive	100	37	83	5	0	0	86	1	72	0	0	0
+	control	100	46	85	2	0	0	86	2	66	0	0	0
	(BP 20)	200: total	83 (41.5)	168 (84.0)	7 (3.5)	0 (0.0)	0 (0.0)	172 (86.0)	3	69	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate.

mammalian cells. Also, bismuth showed positive results in the chromosomal aberration test.

A number of metals and their compounds are known to have genotoxicity or carcinogenicity, and various mechanisms, such as induction of oxidative stress, DNA repair modulation, or disturbances of signal transduction pathways have been suggested as the possible causes[8]. For example, inorganic lead compounds were classified as "probably carcinogenic to humans (Group 2A)" by the International Agency for Research on Cancer (IARC), and induction of oxidative stress and DNA repair inhibition are thought to be the possible mechanisms leading to carcinogenesis[9]. Gastaldo *et al*. reported that lead nitrate exposure resulted in formation of late DNA double-strand breaks and inhibition of non-homologous end-joining repair process[10]. However, in the present study, genotoxicity was not observed for metallic lead. Usually, most carcinogenic metals show weak mutagenicity in mammalian cells and inactivity in bacterial assays. The reason for this is thought to be that the metals act as the enhancers of other mutagenic agents[8]. Thus, further studies to confirm the detailed mechanisms leading to genotoxicity might be needed for metallic lead to reach a conclusion.

Antimony showed genotoxicity in the present study. Although negative results have generally been observed in non-mammalian genotoxicity tests using both trivalent and pentavalent antimony compounds, several studies have reported positive results for *in vitro* experiments in mammalian test systems using only trivalent antimony[11–15]. Thus, our positive results regarding metallic antimony were not incompatible with these preceding studies. Similar to lead, both excess production of active oxygen species and interference with the DNA repair system are thought to be the possible mechanisms of the genotoxicity of antimony[13].

a-2) Lead, pulse treatment (confirmation test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoredupli-cations	Total aberrant cells (%)
	Negative	100	0	0	0	0	0	0	0	93	0	0	0
+	control	100	0	0	0	0	0	0	0	107	0	0	0
	(CMC-Na)	200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	1	0	0	1	0	101	1	0	1
+	250P	100	1	1	0	0	0	1	0	105	0	0	0
		200: total	2 (1.0)	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.0)	0	103	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	0	0	0	0	0	0	108	0	0	0
+	500P	100	0	0	0	0	0	0	0	99	0	0	0
		200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	104	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	0	0	0	0	0	0	89	0	0	0
+	750P	100	0	1	0	0	0	1	0	109	0	0	0
		200: total	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	99	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	0	0	0	0	0	0	109	0	0	0
+	1,000P	100	0	0	0	0	0	0	0	99	0	0	0
		200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	104	0 (0.0)	0 (0.0)	0 (0.0)
	Positive	100	28	87	0	0	0	88	1	83	0	1	1
+	control	100	15	82	0	1	0	86	2	70	0	0	0
	(BP 20)	200: total	43 (21.5)	169 (84.5)	0 (0.0)	1 (0.5)	0 (0.0)	174 (87.0)	3	77	0 (0.0)	1 (0.5)	1 (0.5)

a-3) Lead, continuous treatment

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoredupli-cations	Total aberrant cells (%)
	Negative	100	1	0	0	0	0	1	0	95	0	0	0
–	control	100	1	0	0	0	0	1	0	105	0	0	0
	(CMC-Na)	200: total	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	0	0	1	0	88	0	0	0
–	250P	100	0	0	0	0	0	0	0	87	0	0	0
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	88	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	3	0	0	4	0	94	0	0	0
–	500P	100	2	0	0	0	0	2	0	96	0	0	0
		200: total	3 (1.5)	0 (0.0)	3 (1.5)	0 (0.0)	0 (0.0)	6 (3.0)	0	95	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	1	0	0	0	1	0	88	0	0	0
–	1,000P	100	0	0	0	0	0	0	0	77	0	0	0
		200: total	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	82	0 (0.0)	0 (0.0)	0 (0.0)
	Positive	100	27	18	1	0	0	40	3	103	0	0	0
–	control	100	29	9	0	0	0	37	0	80	0	0	0
	(MMC 0.03)	200: total	56 (28.0)	27 (13.5)	1 (0.5)	0 (0.0)	0 (0.0)	77 (38.5)	3	91	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate.

However, there have been few studies attempting to clarify the mechanism of antimony. With regard to humans, Cavallo *et al*. showed that oxidative DNA damage might be involved in the genotoxicity of antimony in a study including 23 male workers who handled materials containing antimony[16]. In addition, antimony trioxide was classified as possibly carcinogenic to humans (Group 2B), but antimony trisulfide was not classifiable as to its carcinogenicity to humans (Group 3) by IARC[17], based on animal experiments. Nevertheless, epidemiological studies of humans[18, 19] have not yet proven the carcinogenic effects

Table 6. Aberrant cell number in chromosomal aberration test (continued)

b-1) Bismuth, pulse treatment (main test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	2	1	0	0	0	3	1	97	0	0	0
–	control	100	2	0	0	0	0	2	1	103	1	0	1
	(CMC-Na)	200: total	4 (2.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	5 (2.5)	2	100	1 (0.5)	0 (0.0)	1 (0.5)
		100	1	0	0	0	0	1	0	94	1	0	1
–	625P	100	2	0	0	0	0	2	0	80	0	0	0
		200: total	3 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	0	87	1 (0.5)	0 (0.0)	1 (0.5)
		100	1	0	0	0	0	1	0	80	0	0	0
–	1,250P	100	0	0	0	0	0	0	0	115	1	0	1
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	97	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	1	0	0	0	1	0	95	0	0	0
–	2,500P	100	2	0	0	0	0	2	0	75	0	0	0
		200: total	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	0	85	0 (0.0)	0 (0.0)	0 (0.0)
		100	2	1	2	0	0	4	0	61	0	0	0
–	5,000P	100	4	0	0	0	0	4	0	56	0	0	0
		200: total	6 (3.0)	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	8 (4.0)	0	58	0 (0.0)	0 (0.0)	0 (0.0)
	Positive	100	38	26	2	0	0	54	0	80	0	0	0
–	control	100	33	31	0	0	0	52	0	85	0	0	0
	(MMC 0.1)	200: total	71 (35.5)	57 (28.5)	2 (1.0)	0 (0.0)	0 (0.0)	106 (53.0)	0	83	0 (0.0)	0 (0.0)	0 (0.0)
	Negative	100	0	0	0	0	0	0	1	116	0	0	0
+	control	100	0	0	0	0	0	0	0	84	0	0	0
	(CMC-Na)	200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	2	2	0	0	0	4	0	93	0	0	0
+	1,250P	100	3	1	0	0	0	4	0	98	1	0	1
		200: total	5 (2.5)	3 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	8 (4.0)	0	95	1 (0.5)	0 (0.0)	1 (0.5)
		100	3	1	1	0	0	4	0	74	0	1	1
+	2,500P	100	4	2	1	0	0	6	0	52	0	1	1
		200: total	7 (3.5)	3 (1.5)	2 (1.0)	0 (0.0)	0 (0.0)	10 (5.0)	0	63	0 (0.0)	2 (1.0)	2 (1.0)
		100	4	2	2	0	0	6	0	55	1	4	5
+	5,000P	100	5	4	1	0	0	7	0	99	2	2	4
		200: total	9 (4.5)	6 (3.0)	3 (1.5)	0 (0.0)	0 (0.0)	13 (6.5)	0	77	3 (1.5)	6 (3.0)	9 (4.5)
	Positive	100	47	78	0	0	0	82	0	39	0	0	0
+	control	100	37	83	0	0	0	85	0	58	0	0	0
	(BP 20)	200: total	84 (42.0)	161 (80.5)	0 (0.0)	0 (0.0)	0 (0.0)	167 (83.5)	0	48	0 (0.0)	0 (0.0)	0 (0.0)

b-2) Bismuth, pulse treatment (confirmation test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	0	0	0	0	0	0	0	102	0	0	0
+	control	100	0	0	0	0	0	0	0	98	0	0	0
	(CMC-Na)	200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	0	0	1	1	92	0	0	0
+	1,250P	100	1	1	0	0	0	1	0	79	1	0	1
		200: total	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	1	85	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	0	1	0	0	0	0	66	0	0	0
+	2,500P	100	1	1	1	0	0	1	0	82	1	2	3
		200: total	1 (0.5)	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	74	1 (0.5)	2 (1.0)	3 (1.5)
		100	4	2	2	0	0	5	0	65	0	2	2
+	5,000P	100	6	4	1	0	0	7	0	74	0	1	1
		200: total	10 (5.0)	6 (3.0)	3 (1.5)	0 (0.0)	0 (0.0)	12 (6.0)	0	69	0 (0.0)	3 (1.5)	3 (1.5)
	Positive	100	21	66	0	0	0	74	0	58	0	0	0
+	control	100	32	72	0	0	0	73	0	64	0	0	0
	(BP 20)	200: total	53 (26.5)	138 (69.0)	0 (0.0)	0 (0.0)	0 (0.0)	147 (73.5)	0	61	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate.

Table 6. Aberrant cell number in chromosomal aberration test (continued)

c-1) Indium, pulse treatment (main test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	1	0	0	0	0	1	0	103	0	0	0
–	control	100	0	0	1	0	0	1	1	97	0	0	0
	(CMC-Na)	200: total	1 (0.5)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.0)	1	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	2	0	1	0	0	2	1	66	0	0	0
–	156	100	1	0	1	0	0	2	1	76	0	0	0
		200: total	3 (1.5)	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)	4 (2.0)	2	71	0 (0.0)	0 (0.0)	0 (0.0)
		100	2	0	0	0	0	2	0	72	0	0	0
–	313	100	1	1	1	0	0	3	1	74	0	0	0
		200: total	3 (1.5)	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	5 (2.5)	1	73	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	0	0	1	0	49	0	0	0
–	625P	100	1	2	0	0	0	3	1	47	0	0	0
		200: total	2 (1.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)	1	48	0 (0.0)	0 (0.0)	0 (0.0)
		100								31			
–	1,250P	100	TOX	TOX	TOX	TOX	TOX	TOX	TOX	34	TOX	TOX	TOX
		200: total								32			
	Positive	100	31	41	1	0	0	65	3	63	0	0	0
–	control	100	34	46	1	0	0	70	3	86	0	0	0
	(MMC 0.1)	200: total	65 (32.5)	87 (43.5)	2 (1.0)	0 (0.0)	0 (0.0)	135 (67.5)	6	74	0 (0.0)	0 (0.0)	0 (0.0)
	Negative	100	0	0	1	0	0	1	0	103	0	0	0
+	control	100	0	0	1	0	0	1	0	97	0	0	0
	(CMC-Na)	200: total	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)	2 (1.0)	0	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	2	0	0	0	2	0	78	0	0	0
+	625P	100	0	0	0	0	0	0	0	101	0	0	0
		200: total	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0	89	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	0	1	0	0	1	2	98	0	0	0
+	1,250P	100	0	1	0	0	0	1	0	83	0	0	0
		200: total	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.0)	2	90	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	1	0	2	0	72	0	0	0
+	2,500P	100	1	1	0	0	0	2	0	75	0	0	0
		200: total	2 (1.0)	1 (0.5)	0 (0.0)	1 (0.5)	0 (0.0)	4 (2.0)	0	73	0 (0.0)	0 (0.0)	0 (0.0)
		100								62			
+	5,000P	100	TOX	TOX	TOX	TOX	TOX	TOX	TOX	64	TOX	TOX	TOX
		200: total								63			
	Positive	100	7	80	0	1	0	83	2	66	0	0	0
+	control	100	11	75	1	0	0	79	0	68	0	0	0
	(BP 20)	200: total	18 (9.0)	155 (77.5)	1 (0.5)	1 (0.5)	0 (0.0)	162 (81.0)	2	67	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate. TOX: The specimen had less than 50 metaphase cells per plate because of cell toxicity.

of antimony.

Bismuth also showed clastogenic potential in the chromosomal aberration test in the present study. In a previous study performed by von Recklinghausen *et al*., it was reported that methylbismuth had a genotoxic effect on human lymphocytes, and that inhibition of the DNA repair system might be the mechanism leading to DNA damage[20]. It was also mentioned that methylbismuth had much stronger cytotoxic and genotoxic effects on human cells than did other bismuth compounds (bismuth citrate and bismuth glutathione), and the results suggest each bismuth compound might have a unique influence on human cells. Metallic bismuth was examined in the present study, and our results show that it may have genotoxic effects.

Indium has been used in the microelectronics industry in recent years, and the toxicity of inhaled indium has been demonstrated in several reports[21–26]. In a previous report, we showed that orally administered indium did not have any toxic effect on the general condition of rats[3], but there is no previous study describing the genotoxicity of indium. This study is the first study to show that indium

c-2) Indium, continuous treatment

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	0	0	0	0	0	0	0	107	0	0	0
–	control	100	1	0	0	0	0	1	1	93	0	0	0
	(CMC-Na)	200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	1	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	0	0	0	0	0	0	133	0	0	0
–	125	100	1	0	0	0	0	1	0	80	0	0	0
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	106	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	2	0	3	0	74	0	0	0
–	250	100	1	0	0	0	0	1	1	86	0	0	0
		200: total	2 (1.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)	4 (2.0)	1	80	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	1	0	2	1	76	0	0	0
–	500P	100	0	0	0	0	0	0	0	57	0	0	0
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	2 (1.0)	1	66	0 (0.0)	0 (0.0)	0 (0.0)
		100								39			
–	1,000P	100	TOX	TOX	TOX	TOX	TOX	TOX	TOX	63	TOX	TOX	TOX
		200: total								51			
	Positive	100	17	17	0	2	0	32	4	96	0	0	0
–	control	100	12	13	0	0	0	23	4	87	0	0	0
	(MMC 0.03)	200: total	29 (14.5)	30 (15.0)	0 (0.0)	2 (1.0)	0 (0.0)	55 (27.5)	8	91	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate. TOX: The specimen had less than 50 metaphase cells per plate because of cell toxicity.

is not genotoxic. However, the average particle size of indium was larger than the cell size used in the present study, because it was impossible to make smaller indium particles due to a feature of metallic indium. Therefore, it might be difficult to interpret the results of the chromosomal aberration tests for indium without identifying its absorption rate by the cells.

Silver has been widely used in dishes, instruments, and dental inlays due to its benign nature. Negative results were obtained for silver in the present study, and the result reconfirmed the benignity of silver.

As in our study, a combination of *in vitro* tests, namely, both tests for mutagenicity in bacteria and clastogenicity in cultured mammalian cells, are routinely performed to explore the genotoxicity of drug candidates or chemicals[27, 28]. In addition, *in vivo* studies are needed to reach a consensus. It is difficult to conclusively evaluate genotoxicity, because we have to evaluate the toxicity without consideration for absorption, distribution, and metabolism in the actual human body. Additionally, because the specificity of mammalian mutagenicity tests are below 50%[29], the possibility of false positives cannot be ignored at all times. Precipitation of metals in this study also needs to be considered, because if the treatment concentrations were insufficient, they could have lead to negative results. Although bismuth showed genotoxicity in the chromosomal aberration tests, phagocytosis of the cultured cells might have had considerable influence on the positive results, as bismuth was partially precipitated. Thus, further studies, specifically *in vivo* studies, are essential if we are to clarify the genotoxicity of the heavy metals discussed in the present study.

In conclusion, antimony and bismuth had genotoxic effects in the present study. Although further studies are needed, this shows that we have to pay close attention to the toxic effects of these materials when they are used.

Acknowledgments: This work was supported financially by the IMS (Intelligent Manufacturing Systems) Promotion Center, Manufacturing Science and Technology Center. Study numbers of the bacterial reverse mutation tests are B000638 for lead, B000630 for bismuth, B000634 for indium, B000626 for silver, and B010825 for antimony. Those for the chromosomal aberration tests in cultured mammalian cells are B000639 for lead, B000631 for bismuth, B000635 for indium, B000627 for silver, and B010826 for antimony.

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Table 6. Aberrant cell number in chromosomal aberration test (continued)

d-1) Silver, pulse treatment (main test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	1	0	0	0	0	1	1	97	0	0	0
–	control	100	0	0	1	1	0	2	0	103	0	0	0
	(CMC-Na)	200: total	1 (0.5)	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	3 (1.5)	1	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	2	0	0	0	0	2	0	111	0	0	0
–	1,250P	100	0	0	0	0	0	0	0	110	0	0	0
		200: total	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0	111	0 (0.0)	0 (0.0)	0 (0.0)
		100	1	0	0	0	0	1	0	87	1	0	1
–	2,500P	100	0	0	1	0	0	1	0	89	2	0	2
		200: total	1 (0.5)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.0)	0	88	3 (1.5)	0 (0.0)	3 (1.5)
		100	0	0	0	0	0	0	0	63	0	0	0
–	5,000P	100	1	0	0	0	0	1	0	56	0	0	0
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	60	0 (0.0)	0 (0.0)	0 (0.0)
	Positive	100	40	33	0	0	0	60	1	114	0	0	0
–	control	100	35	30	1	0	0	56	0	65	0	0	0
	(MMC 0.1)	200: total	75 (37.5)	63 (31.5)	1 (0.5)	0 (0.0)	0 (0.0)	116 (58.0)	1	89	0 (0.0)	0 (0.0)	0 (0.0)
	Negative	100	0	0	0	0	0	0	0	106	1	0	1
+	control	100	0	0	0	0	0	0	0	94	0	0	0
	(CMC-Na)	200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	100	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	0	1	0	0	1	0	74	1	0	1
+	1,250P	100	1	0	0	1	0	2	0	67	1	0	1
		200: total	1 (0.5)	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	3 (1.5)	0	71	2 (1.0)	0 (0.0)	2 (1.0)
		100	0	0	0	0	0	0	0	83	1	0	1
+	2,500P	100	0	0	0	0	0	0	0	83	0	0	0
		200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	83	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	0	0	0	0	0	0	60	2	0	2
+	5,000P	100	0	0	0	0	0	0	0	69	1	0	1
		200: total	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	64	3 (1.5)	0 (0.0)	3 (1.5)
	Positive	100	11	50	0	0	0	50	0	59	0	0	0
+	control	100	13	57	0	0	0	62	0	52	0	0	0
	(BP 20)	200: total	24 (12.0)	107 (53.5)	0 (0.0)	0 (0.0)	0 (0.0)	112 (56.0)	0	55	0 (0.0)	0 (0.0)	0 (0.0)

d-2) Silver, continuous treatment

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	1	0	0	0	0	1	0	88	0	0	0
–	control	100	1	0	0	0	0	1	0	113	2	0	2
	(CMC-Na)	200: total	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0	100	2 (1.0)	0 (0.0)	2 (1.0)
		100	1	0	1	1	0	2	0	92	1	0	1
–	1,250P	100	1	0	0	0	0	1	0	104	1	0	1
		200: total	2 (1.0)	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	3 (1.5)	0	98	2 (1.0)	0 (0.0)	2 (1.0)
		100	0	0	0	0	0	0	0	85	0	1	1
–	2,500P	100	1	0	0	0	0	1	0	82	2	0	2
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	83	2 (1.0)	1 (0.5)	3 (1.5)
		100	3	0	0	0	0	3	0	46	2	1	3
–	5,000P	100	1	0	0	0	0	1	1	64	2	0	2
		200: total	4 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)	1	55	4 (2.0)	1 (0.5)	5 (2.5)
	Positive	100	25	4	1	0	0	30	0	94	0	0	0
–	control	100	19	6	0	0	0	23	0	71	0	0	0
	(MMC 0.03)	200: total	44 (22.0)	10 (5.0)	1 (0.5)	0 (0.0)	0 (0.0)	53 (26.5)	0	82	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate.

Table 6. Aberrant cell number in chromosomal aberration test (continued)

e) Antimony, pulse treatment (main test)

S9 mix	Concentration (μg/ml)	Number of cells	Number of structurally aberrant cells (%)						Number of gap	Cell growth index (%)	Number of numerically aberrant cells (%)		
			Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Fragments	Total aberrant cells (%)			Polyploids	Endoreduplications	Total aberrant cells (%)
	Negative	100	0	0	0	0	0	0	0	104	0	0	0
–	control	100	1	0	0	0	0	1	0	96	0	0	0
	(CMC-Na)	200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	2	0	0	0	2	0	106	1	0	1
–	12.5	100	2	0	0	0	0	2	1	106	0	1	1
		200: total	2 (1.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)	1	106	1 (0.5)	1 (0.5)	2 (1.0)
		100	0	1	0	0	0	1	0	79	1	4	5
–	25	100	1	1	0	0	0	2	1	70	0	2	2
		200: total	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	1	75	1 (0.5)	6 (3.0)	7 (3.5)
		100	18	9	0	0	0	20	0	50	1	5	6
–	50	100	10	6	1	0	1	13	0	56	1	13	14
		200: total	28 (14.0)	15 (7.5)	1 (0.5)	0 (0.0)	1 (0.5)	33 (16.5)	0	53	2 (1.0)	18 (9.0)	20 (10.0)
		100	39	20	0	0	2	46	1	28	0	5	5
–	100	100	37	27	0	1	6	50	1	28	1	4	5
		200: total	76 (38.0)	47 (23.5)	0 (0.0)	1 (0.5)	8 (4.0)	96 (48.0)	2	28	1 (0.5)	9 (4.5)	10 (5.0)
		100								18			
–	200	100	TOX	TOX	TOX	TOX	TOX	TOX	TOX	14	TOX	TOX	TOX
		200: total								16			
	Positive	100	21	19	1	0	0	38	0	–	0	0	0
–	control	100	15	16	0	0	0	27	0	–	0	0	0
	(MMC 0.1)	200: total	36 (18.0)	35 (17.5)	1 (0.5)	0 (0.0)	0 (0.0)	65 (32.5)	0	–	0 (0.0)	0 (0.0)	0 (0.0)
	Negative	100	0	0	0	0	0	0	1	109	0	0	0
+	control	100	0	1	1	0	0	2	0	91	0	0	0
	(CMC-Na)	200: total	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	2 (1.0)	1	100	0 (0.0)	0 (0.0)	0 (0.0)
		100	0	0	0	0	0	0	0	109	1	0	1
+	6.25	100	1	0	0	0	0	1	0	105	0	0	0
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	107	1 (0.5)	0 (0.0)	1 (0.5)
		100	0	0	0	0	0	0	0	92	0	1	1
+	12.5	100	1	0	0	0	0	1	0	103	1	0	1
		200: total	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0	97	1 (0.5)	1 (0.5)	2 (1.0)
		100	6	8	2	2	0	14	1	86	2	25	27
+	25	100	5	9	1	0	1	14	1	91	1	20	21
		200: total	11 (5.5)	17 (8.5)	3 (1.5)	2 (1.0)	1 (0.5)	28 (14.0)	2	89	3 (1.5)	45 (22.5)	48 (24.0)
		100	28	25	0	0	0	35	1	50	1	15	16
+	50	55	15	14	1	0	2	21	0	43	3	8	11
		155: total	43 (27.7)	39 (25.2)	1 (0.6)	0 (0.0)	2 (1.3)	56 (36.1)	1	46	4 (2.0)	23 (14.8)	27 (17.4)
		100								36			
+	100	100	TOX	TOX	TOX	TOX	TOX	TOX	TOX	34	TOX	TOX	TOX
		200: total								35			
		100								21			
+	200	100	TOX	TOX	TOX	TOX	TOX	TOX	TOX	27	TOX	TOX	TOX
		200: total								24			
	Positive	100	21	69	0	1	0	74	0	–	0	0	0
+	control	100	16	72	0	0	0	73	2	–	0	0	0
	(BP 20)	200: total	37 (18.5)	141 (70.5)	0 (0.0)	1 (0.5)	0 (0.0)	147 (73.5)	2	–	0 (0.0)	0 (0.0)	0 (0.0)

P: At the end of the treatment (6-h treatment), some of the test substances precipitated and adhered to the bottom inside the plate. TOX: The specimen had less than 50 metaphase cells per plate because of cell toxicity.

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