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Indium Chloride-induced Micronuclei in *In Vivo* and *In Vitro* Experimental Systems

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Abstract: Indium Chloride-induced Micronuclei in *In Vivo* and *In Vitro* Experimental Systems: Ryo TAKAGI, *et al*. Department of Public Health and Environmental Medicine, Jikei University School of Medicine—Objectives: The aim of this study was to investigate the genotoxic effects of indium trichloride ($InCl_3 \cdot 4H_2O$; $InCl_3$) using the *in vivo* bone marrow micronucleus test and the *in vitro* CHL/IU cell micronucleus test. **Method:** BALB/c mice were administered a single intraperitoneal (i.p.) injection of $InCl_3$ at a dose 0.625, 1.25, 2.5, 5, or 10 mg/kg b.w. The frequency of micronuclei, the ratio of polychromatic erythrocytes to normochromatic erythrocytes (P/N ratio) and body weight gain were determined 24 h after administration of the $InCl_3$. In the *in vitro* micronucleus test, CHL/IU cells were treated continuously for 24, 48, or 72 h in the absence of S9mix (the continuous treatment method) and/or for 6 h with or without S9 mix followed by an 18, 42 or 66 h recovery time (the short time treatment method). The frequency of micronuclei was determined at the end of each culture period. **Results:** The frequency of micronuclei induced by $InCl_3$ increased in the *in vivo* erythroblast-erythrocyte micronucleus test using BALB/c mice at doses of 2.5 and 5 mg/kg b.w. The P/N ratio, a marker of bone marrow toxicity, decreased significantly following the injection of $InCl_3$. Body weight gain was also inhibited by $InCl_3$. $InCl_3$ induced micronuclei in the CHL/IU cell micronucleus test in both the continuous treatment method and the short time treatment method, both with and without S9mix. **Conclusions:** These results suggest that $InCl_3$ has a genotoxic effect on mammalian cells both *in vivo* and *in vitro*.
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Indium compounds, including indium trichloride, are used for the diagnosis of myelopoiesis, and in ceramics for the manufacture of transparent conductive films for liquid crystal and plasma flat-panel displays. The toxicity of these compounds in humans has been attracting increasing attention.

There is limited information available concerning indium compounds, and they were thought to be safe until the mid-1990's. However, there have been cases of death due to interstitial pneumonia that are thought to have been caused by indium-tin oxide (ITO) inhalation[1], and interstitial lung disease has been reported in workers handling ITO [1–3]. A recent study reported the carcinogenicity of indium (III) phosphide (InP), which is a compound used in semiconductors, and severe lung damage has also been reported to be associated with other indium compounds as well as InP[4].

The demand for indium in Japan in 2007 was 1,146 tons, which accounted for 85% of the world demand[5]. Moreover, 80% of all demand is for ITO[5]. The use of ITO in the production of flat-panel displays poses a significant health issue, especially in Japan.

It is known that indium trichloride accumulates in the erythroblasts[6, 7]. This property makes it useful as a diagnostic method for myelopoiesis[7]. Indium trichloride is currently not thought to be a carcinogen like InP, but no bone marrow erythroblast-erythrocyte micronucleus tests have been reported for the compound. The bone marrow erythroblast-erythrocyte micronucleus test, an *in vivo* screening assay for detecting mutagens and carcinogens, is widely used in cytogenetic studies[8, 9]. Micronuclei commonly arise from acentric chromosomal fragments that are not included in one of the daughter nuclei following erythroblast division[10].

The aim of this study was to clarify the genotoxic activity of $InCl_3$ using an *in vivo* bone marrow erythroblast-erythrocyte micronucleus test and the *in vitro* CHL/IU cell

line micronucleus test.

Materials and Methods

Chemicals

$InCl_3 \cdot 4H_2O$ ($InCl_3$: CAS; 10025-82-8) was obtained from Aldrich (WI, USA). Fetal bovine serum, bovine serum, trypsin, and glucose-6-phosphate were supplied by GIBCO (NY, USA). Eagle's minimum essential medium was supplied by Sigma (MN, USA). NADH was obtained from Roche (Mannheim, Germany). NADPH was purchased from WAKO Pure Chem (Osaka, Japan). S9 was purchased from Kikkoman (Noda, Japan).

In vivo micronucleus test

1. Experimental animals

BALB/c AnNCrlCrlj male mice, at 7 wk of age and with a body weight (b.w.) between 22 and 27 g, were obtained from Charles River Japan (Yokohama, Kanagawa, Japan). The mice were kept in an animal room that was maintained at a constant temperature and humidity (24 ± 1°C and 50 ± 5%, respectively) under a 12-hour light-dark cycle. The mice were given water and a CRF-1 diet supplied by Charles River Japan *ad libitum*. Each group used in the micronucleus test consisted of five male animals. These experiments were performed following the Guidelines on Animal Experimentation of Jikei University.

2. Micronucleus test

①Induction of micronuclei by $InCl_3$

$InCl_3$ was dissolved in phosphate-buffered saline (PBS: pH 7.2, Sigma, St. Louis, MO, USA). Groups of five male mice were intraperitoneally (i.p.) administered $InCl_3$ once at a dose of 0.625, 1.25, 2.5, 5, or 10 mg/kg b.w. The maximum dose was two times higher than the LD_{50} (5 mg/kg) value of $InCl_3$. The mice were sacrificed by cervical dislocation 24 h after the administration of $InCl_3$. Bone marrow cell smears were prepared after flushing them out with fetal bovine serum.

②Bone marrow preparation and evaluation of results

Bone marrow smears were stained with May Grünwald-Giemsa solution (1/150M Sörensen's phosphate buffer solution (pH 6.4)) as described by Schmid[11]. The number of micronucleated polychromatic erythrocytes (MPCE) in 1,000 polychromatic erythrocytes (PCE) and the ratio of PCE to normochromatic erythrocytes (P/N ratio; a marker of bone marrow toxicity[12]) per animal were counted under a light microscope at 1,000x magnification. The data for micronucleus induction were statistically evaluated using Fisher's exact test. The dose-response relationship was evaluated using the Cochran-Armitage trend test. The data for the P/N ratio were also statistically analyzed using Student's *t*-test.

3. *In vitro* micronucleus test

①Cells

CHL/IU cells were obtained from the National Institute of Health Science (Japan). They were maintained in Eagle's minimum essential medium (medium) supplemented with 10% heat inactivated (56°C for 30 min) calf serum.

②Rat liver S9 and preparation of S9mix

Rat liver S9 fractions prepared from phenobarbital- and 5, 6-benzoflavone-induced male Sprague-Dawley rats were used for this study. A 1 m*l* aliquot of S9 mix containing 0.1 m*l* S9, 8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol glucose-6-phosphate, 4 μmol NADPH and 4 μmol NADH was used for the study.

③Test chemical treatment and slide preparation

This study was performed as described by Schmid with minor modifications[13, 14]. In the continuous treatment method, cultures of 1×10^5 CHL/IU cells were seeded in 60 mm plastic dishes and changed to fresh medium containing $InCl_3$ on the second day. The cells were incubated for 24, 48, and 72 h in the absence of S9 mix (24, 48, 72 h in the fig). For the short time exposure method, the CHL/IU cells were treated with $InCl_3$ for 6 h with or without S9 mix and then washed 3 times with medium and incubated in medium for 18, 42 or 66 h as a recovery time (6–18 h, 6–42 h, 6–66 h in the fig). The cells were divided into two groups. One group was used to count the cell number using a hemocytometer after trypsinization. The toxic effect (the growth ratio) of $InCl_3$ on CHL/IU cells was calculated as a percentage of the control. The other group of cells was washed 3 times with PBS, and then detached by trypsinization. These cells were washed 3 times with PBS and treated with 75 mM KCl hypotonic solution for 10 min at room temperature. The hypotonized cells were fixed in three changes of 1:3 acetic acid:methanol. Finally, the cells were suspended in methanol containing 1.5% acetic acid and air dried on clean glass slides. The cells were stained with Giemsa solution. These experiments were performed using 3 dishes for each dose.

④Microscopic observation and statistical analysis

Only well-outlined cells with a single main nucleus were counted under a light microscope at 600x magnification. The frequency of micronucleated cells was compared with those of the concurrent negative control by the Cochran-Armitage test with Fisher's exact text.

Results

In vivo micronucleus test

The frequency of micronuclei induced by $InCl_3$ increased in a dose-dependent manner in the *in vivo* bone marrow erythroblast-erythrocyte micronucleus test using BALB/c mice. Significant increases in the micronucleus frequencies ($p<0.05$) were observed at doses of 2.5 and 5 mg/kg of $InCl_3$, and the highest frequency of micronuclei (6.3%) in erythrocytes was observed at a dose of 5 mg/kg (Fig. 1).

The ratio of polychromatic erythrocytes to normochromatic erythrocytes (P/N ratio), a marker of bone

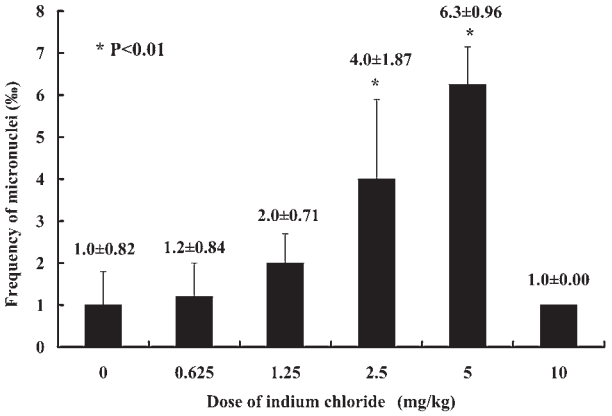

Fig. 1. The induction of micronuclei in mouse bone marrow by injection of $InCl_3$. The data reported are the means ± SD. Each group used in the micronucleus test consisted of five male animals. The statistical analysis was based on the Cochran-Armitage test with Fisher's exact test. The frequency of micronuclei increased dose-dependently (p<0.01) in the $InCl_3$ group.

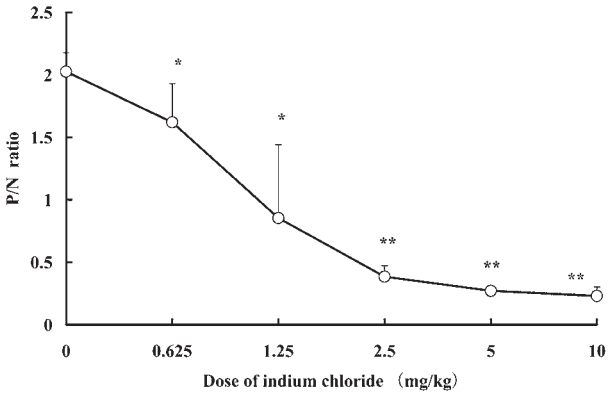

Fig. 2. The effect of $InCl_3$ injection on the P/N ratio. The data reported are the means ± SD. Each group used in the micronucleus test consisted of five male animals. The statistical analysis was based on one way ANOVA with Student's t-test. * p<0.05, **p<0.01.

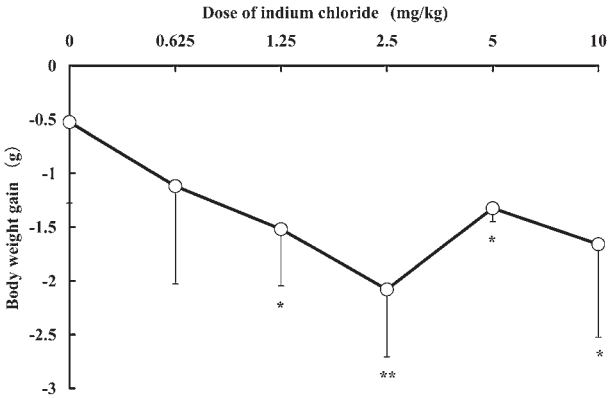

Fig. 3. Body weight gain after the injection of $InCl_3$. The data reported are the means ± SD. Each group used in the micronucleus test consisted of five male animals. Statistical analysis was based on one way ANOVA with Student's t-test. * p<0.05, ** p<0.01.

marrow toxicity, decreased significantly (p<0.05) in a dose-dependent manner after the injection of $InCl_3$ (Fig. 2). In addition, body weight gain was also inhibited significantly (p<0.05) by the injection of $InCl_3$ (Fig. 3).

In vitro CHL/IU micronucleus test

1. Continuous treatment method

Figure 4–1 shows the growth ratio of CHL/IU cells after exposure to $InCl_3$. The growth ratio of CHL/IU when cells were exposed to $InCl_3$ concentrations ranging from 0.3 to 5 μg/m*l* at 48 and 72 h was significantly lower (p<0.001) than that at 24 h. The growth ratio was 27.3, 4.9 and 2.3% at a dose of 5 μg/m*l* of $InCl_3$ after 24, 48 and 72 h of exposure, respectively. The frequency of micronuclei induced by $InCl_3$ increased (p<0.05) significantly at a dose of 5 μg/m*l* after 24 h continuous treatment in the CHL/IU micronucleus test. Similarly, a significant increase in the micronucleus frequency (p<0.05) was observed at doses of 1.25, 2.5, and 5 mg/m*l* of $InCl_3$ after continuous treatment for 48 or 72 h compared with 0 mg/m*l* (Fig. 4–2).

The frequency of micronuclei increased dose-dependently at 24 h (p<0.005), 48 h and 72 h (p<0.001).

2. Short time treatment method

Figure 5–1 shows the growth ratio of CHL/IU cells after short time exposure to $InCl_3$ without S9 mix. The growth ratio of CHL/IU decreased in a dose- and time- dependent manner after exposure to $InCl_3$ (p<0.001). The growth ratios were 29.4, 3.9 and 4.5 % at a dose of 18.75 μg/m*l* of $InCl_3$ at 24, 48 and 72 h exposure, respectively. Significant increases in the micronucleus frequency (p<0.05) were observed at a dose of 9.38 μg/m*l* for 6–18 h (treatment-recovery) without S9 mix (Fig. 5–2). Similarly, significant increases in the micronucleus frequency (p<0.05) were observed at doses ranging from 0.59 to 18.75 μg/m*l* over 6–66 h without S9 mix (Fig. 5–2). The frequency of micronuclei increased dose-dependently (p<0.001) during the 6–66 h time period (Fig. 5–2).

Figure 6–1 shows the growth ratio of CHL/IU cells after short time exposure to $InCl_3$ with the S9 mix. The growth ratio of CHL/IU decreased in a dose- and time-dependent manner after exposure to $InCl_3$ (p<0.001). The growth ratios were 40.5, 11.2 and 3.6% at a dose of 75 μg/m*l* of

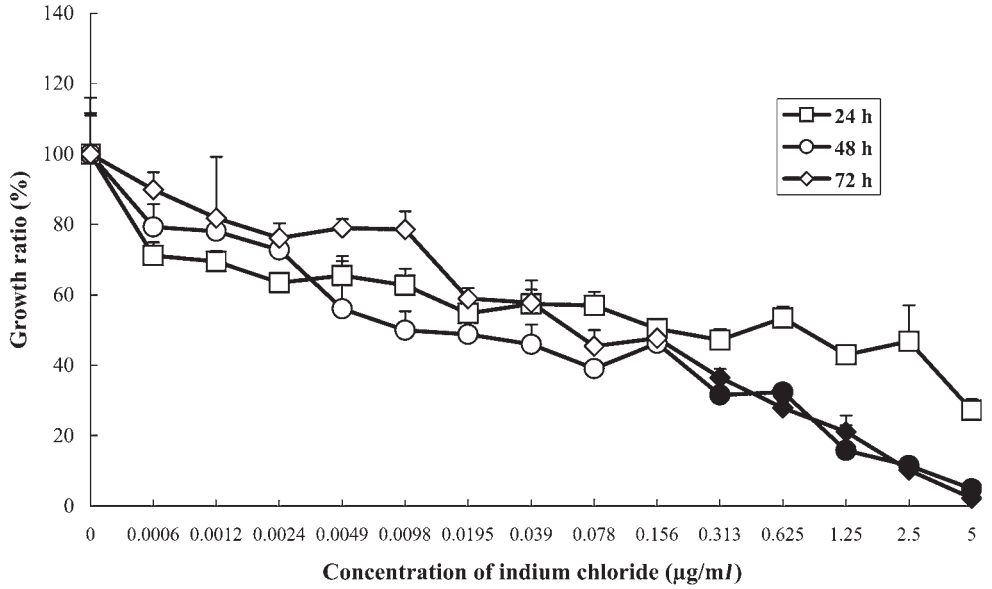

Fig. 4–1. The effect of $InCl_3$ on the growth of CHL/IU cells (continuous treatment method). The data reported are the means ± SD. These experiments were performed twice using 3 dishes for each dose. The statistical analysis was based on one way ANOVA with Student's *t*-test. The % ratio of the control CHL/IU cell number decreased dose-dependently after 24, 48 and 72 h (p<0.01) of treatment. The cell number was markedly decreased (0.313–5 μg/m*l*) after 48 and 72 h of exposure compared with 24 h (●, ◆: p<0.05).

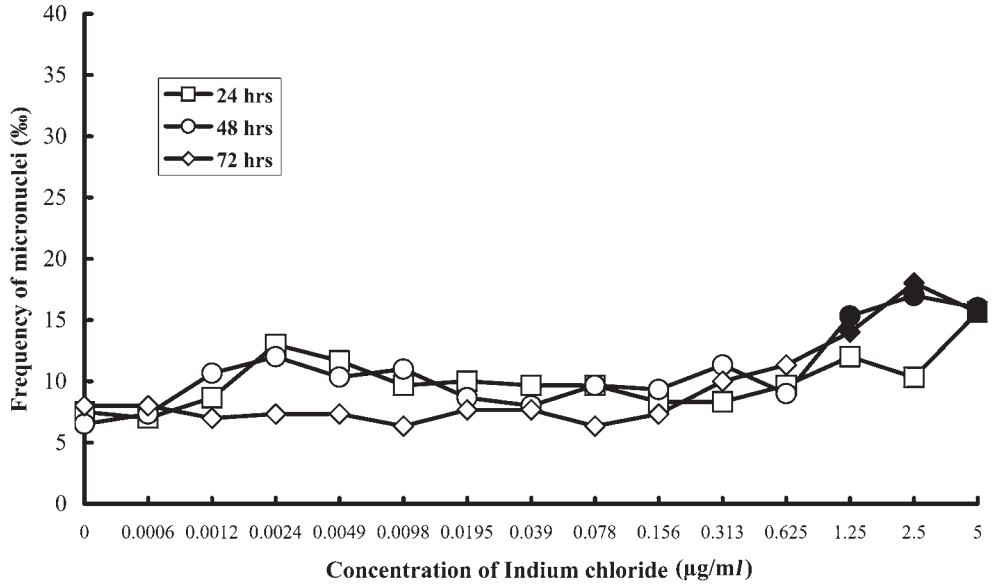

Fig. 4–2. The frequency of micronuclei in CHL/IU cells induced by $InCl_3$ (continuous treatment method). The data reported are the means ± SD. These experiments were performed twice using 3 dishes for each dose. The statistical analysis was based on the Cochran-Armitage test with Fisher's exact test. □, ○, ◇: p>0.05, ■, ●, ◆: p<0.05. The frequency of micronuclei increased dose-dependently after 24 (p<0.005), 48 and 72 h (p<0.001) of exposure, respectively. A significant increase in the micronucleus frequency (p<0.05) was observed at doses of 1.25, 2.5, and 5 mg/m*l* of $InCl_3$ after continuous treatment for 48 or 72 h, compared with 0 mg/m*l*.

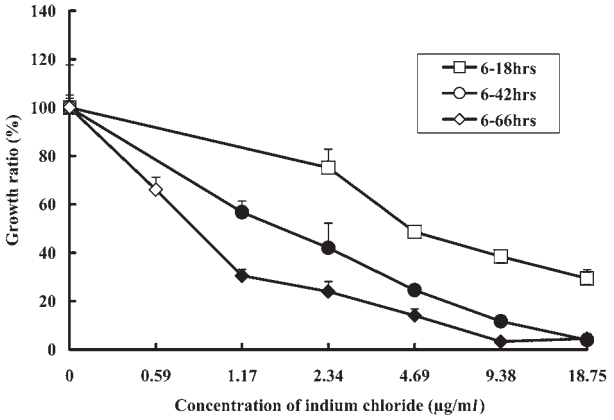

Fig. 5–1. The effect of $InCl_3$ on the growth of CHL/IU cells (short time exposure method without S9 mix). The data reported are the means ± SD. These experiments were performed twice using 3 dishes for each dose. The statistical analysis was based on one way ANOVA with Student's *t*-test. The % ratio of the control CHL/IU cell number decreased recovery time-dependently (18, 42 or 66 h) in the 6–18, 6–42 and 6–66 h groups ($p<0.05$). The cell number was markedly decreased (1.17–18.75 μg/m*l*) in the 6–42 and 6–66 h groups compared with 6–18 group (●, ◆: $p<0.05$).

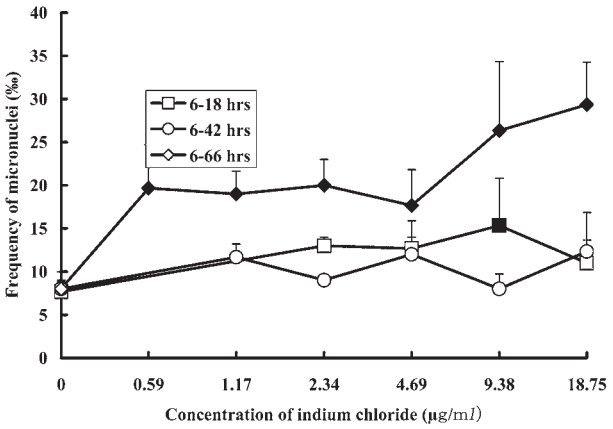

Fig. 5–2. The frequency of micronuclei in CHL/IU cells after exposure to $InCl_3$ (short time treatment method without S9 mix). The data reported are the means ± SD. These experiments were performed repeated twice using 3 dishes for each dose. The statistical analysis was based on the Cochran-Armitage test with Fisher's exact test. □, ○, ◇: $p>0.05$, ■, ●, ◆: $p<0.05$. Significant increases in micronucleus frequency ($p<0.05$) were observed at doses of 0.59–18.75 μg/m*l* for 6–66 h without S9 mix, compared with 0 mg/m*l*. The frequency of micronuclei increased dose-dependently ($p<0.001$) over 6–66 h of exposure.

$InCl_3$ over 6–18, 6–42 and 6–66 h exposure, respectively (Fig. 6–1). Significant increases in the micronucleus frequency ($p<0.05$) were observed at doses of 18.75 μg/ml over 6–18 h (treatment-recovery), 2.34–75 μg/m*l* over 6–42 h and 4.69–18.75 μg/m*l* over 6–66 h with S9 mix, respectively (Fig. 6–2).

The frequency of micronuclei increased dose-dependently ($p<0.001$) over 6–66 h (Fig. 6–2).

3. Comparison of the micronucleus induction intensity

The highest micronucleus frequency per unit concentration (micronucleated cells/μg/m*l*) was 20.1 in the short time exposure method without S9 mix at a dose of 0.59 μg/m*l* over 6–66 h. On the other hand, the micronucleus frequency per unit concentration was 7.1 and 3.3 in the continuous treatment method (1.25 μg/m*l* for 48 h) and in the short time exposure method with S9 mix (2.34 μg/m*l* for 6–42 h), respectively. The highest frequency of micronuclei induced by $InCl_3$ in all experiments was observed at doses of 18.75 μg/m*l* over 6–66 h without S9 mix (29.3% , $p<0.05$).

Discussion

Approximately 80–90% of the indium used in Japan is used in the manufacture of transparent conductive films for liquid crystal and plasma flat-panel displays, with the remaining 10–20% being used in low melting metal bonding agents in CIGS solar batteries[5].

InP, which is a compound used in semiconductors, induces not only severe lung damage, but also cancer[4]. Gottschling *et al.*[15] reported the inhalation of InP causes pulmonary inflammation associated with oxidative stress. They postulated the existence of an inflammation-carcinoma sequence induced by InP inhalation in which chronic, prolonged inflammation is associated with the release of highly reactive oxygen and nitrogen species from inflammatory cells[15]. Moreover, InP is classified in Group 2A (extraordinarily high incidence of malignant neoplasms of the lung in rats and mice, increased incidence of pheochromocytomas in rats, and increased incidence of hepatocellular neoplasms in mice) by the International Agency for Research on Cancer (IARC)[16]. There is a significant increase in micronucleated polychromatic erythrocytes in male, but not in female, mice exposed to InP[4]. These observations suggest that oxidative stress induced by InP has not only genotoxic, but also is tumorigenic, potential.

ITO exposure in the work environment generates interstitial pneumonia[1–3]. Since indium continues to be used in various industries, it is necessary to clarify the carcinogenicity of indium compounds. In this study, we performed *in vivo* and an *in vitro* micronucleus tests to

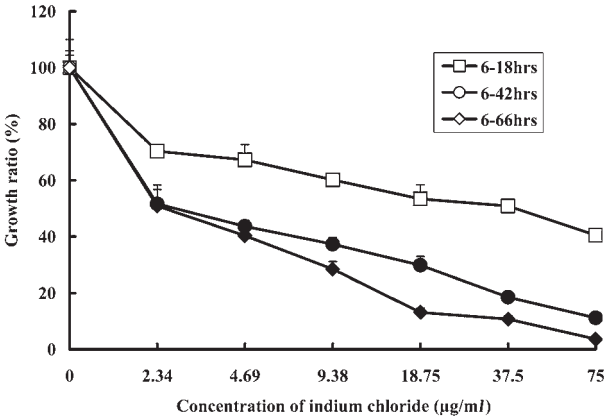

Fig. 6–1. The effect of $InCl_3$ on the growth of CHL/IU cells (short time exposure method with S9 mix). The data reported are the means ± SD. These experiments were performed twice using 3 dishes for each dose. The statistical analysis was based on one way ANOVA with Student's *t*-test. The % ratio of the control CHL/IU cell number decreased recovery time-dependently (18, 42 or 66 h) in the 6–18, 6–42 and 6–66 h groups (p<0.05). The cell number was markedly decreased (2.34–75 μg/m*l*) in the 6–42 and 6–66 h groups compared with 6–18 group (●, ◆: p<0.05).

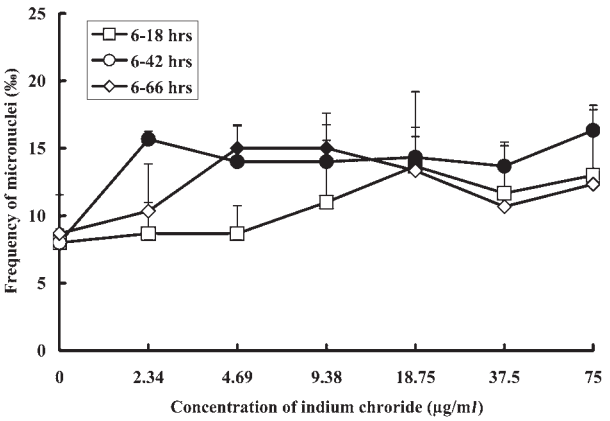

Fig. 6–2. The frequency of micronuclei induced by $InCl_3$ exposure (short time treatment method with S9 mix). The data reported are the means ± SD. These experiments were performed twice using 3 dishes for each dose. The statistical analysis was based on the Cochran-Armitage test with Fisher's exact test. □, ○, ◇: p>0.05, ■, ●, ◆: p<0.05. Significant increases in the micronucleus frequency (p<0.05) were observed at doses of 18.75 μg/m*l* over 6–18 h (treatment-recovery), 2.34–75 μg/m*l* over 6–42 h and 4.69–18.75 μg/m*l* over 6–66 h with S9 mix. The frequency of micronuclei increased dose-dependently (p<0.001) over 6–66 h.

predict the carcinogenicity of indium trichloride. Indium tin oxide (ITO) particles induce an increased frequency of micronuclei in type II pneumocytes *in vivo* but not in lung epithelial cells *in vitro*, thus suggesting the preponderance of a secondary genotoxic mechanism[17]. Lison *et al.*[17] demonstrated in experimental animals that ITO particles are capable of generating hydroxyl radicals via the Fenton reaction, which then cause cytotoxicity in macrophages and trigger an inflammatory lung reaction. These data would explain the pulmonary manifestations reported in workers exposed to ITO[1–3, 18]. The *in vivo* genotoxic potential of ITO particles for lung epithelial cells indicates their capacity to induce lung cancers[17]. These results also show the relationship between free radicals and the genotoxic and/or tumorigenic potential.

Rowlands[19] reported that indium compounds (including indium trichloride) show great potential as reagents in radical reactions. On the other hand, Dusre *et al.*[20] reported that mitomycin C (MMC) induced not only DNA damage (alkylation and DNA DSB) but also stimulated oxy-radical formation. It is reported that ascorbic acid, an antioxidant chemical, decreased the frequency of micronuclei induced by MMC[21]. It is considered that the mechanism of micronucleus induction may therefore involve free radicals.

In the present study, $InCl_3$ showed toxic effects through reduction of body weight and the decrease of the P/N ratio in BALB/c mice. In particular, the decrease in the P/N ratio suggests that $InCl_3$ affects the bone marrow erythropoiesis by inducing chromosome aberration. Suzuki *et al.*[12] suggested that the decrease in the P/N ratio induced by bone marrow suppression may cause an increase of micronuclei *in vivo*. It is generally considered that a chemical which induces micronuclei usually also induces a decrease in the P/N ratio. These phenomena occur because of the inhibition of erythroblast division (suppression of erythropoiesis) by the chemically-induced chromosome aberration. The frequency of micronuclei at a dose of 10 mg/kg in our study was lower than that of 5 mg/kg. It is considered that micronuclei did not increase because the strong toxic effect of 10 mg/kg $InCl_3$ suppressed erythroblast division.

It is known that $InCl_3$ accumulates in bone marrow erythroblasts, thus making the compound useful in the diagnosis of myelopoiesis[6], and this results suggests that $InCl_3$ might induce micronuclei in erythroblasts.

A number of studies have shown that the administration of soluble indium as $InCl_3$, or InAs particles, lead to the potent induction of heme oxygenase, which is the rate-limiting enzyme in the heme degradation pathway. The induction of this enzyme is used as a molecular marker of oxidative stress[16, 22–24]. The primary genotoxic mechanism of $InCl_3$ may thus involve oxidative stress, similar to InP[15] and ITO[17]. However, precancerous change in the lung

was caused only by InAs when indium arsenide, gallium arsenic or arsenious acid were administered intratracheally to hamsters[25]. These results also suggest that indium possibly induces carcinogenesis and mutagenesis *in vivo*.

Similarly, $InCl_3$ induced micronuclei in the *in vitro* CHL/IU micronucleus test. The positive result in the CHL/IU test may have been due to the chemical structure, water-soluble properties and/or the direct exposure of cultured cells. The highest frequency of micronuclei was observed in the short time treatment method without S9 mix. For $InCl_3$, the frequency of micronuclei became higher under exposure to a high concentration for a short time and using a long recovery time. The S9 mix may decrease the frequency of micronuclei. Because $InCl_3$ is changed into $In(OH)_3$ in neutral and alkali solutions[26], $In(OH)_3$ may induce micronuclei under this experimental condition.

The growth of CHL/IU cells was inhibited dose- and time- dependently after treatment with $InCl_3$. This is likely because the genotoxic effects of $InCl_3$ cause the micronucleus induction to occur in tandem with the extent of growth inhibition of the CHL/IU cells.

Based on the *in vivo* and *in vitro* genotoxic test results, we hypothesized that the indium compounds most likely exert their genotoxic effects through accumulation of indium in target cells.

In the Ames test, $InCl_3$ did not induce mutagenic activity with and without S9mix in the TA100, TA2637, TA94, TA98, WP2*uvr*A$^+$ and WP2*uvr*A$^-$[27] strains. These results suggest that $InCl_3$ may not induce mutagenicity in a bacterial mutation test system[27], however, it did induce micronuclei in mammalian cells in both *in vivo* and *in vitro* test systems in our study. Further studies are therefore needed to clarify the relationships between the mechanisms of tumor inducibility and micronucleus inducibility by $InCl_3$.

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