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 (InCl₃·4H₂O; InCl₃) 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 InCl₃ 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 InCl₃. 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 InCl₃ 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 InCl₃. Body weight gain was also inhibited by InCl₃. InCl₃ 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 InCl₃ has a genotoxic effect on mammalian cells both in vivo and in vitro. (J Occup Health 2011; 53: 102–109)

Key words: Bone marrow, CHL, Indium, Micronuclei

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) inhalation¹, 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 InP⁴.

The demand for indium in Japan in 2007 was 1,146 tons, which accounted for 85% of the world demand⁵. Moreover, 80% of all demand is for ITO⁵. 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 erythroblasts⁶, ⁷. This property makes it useful as a diagnostic method for myelopoiesis⁷. 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 studies⁸, ⁹. Micronuclei commonly arise from acentric chromosomal fragments that are not included in one of the daughter nuclei following erythroblast division⁹.

The aim of this study was to clarify the genotoxic activity of InCl₃ using an in vivo bone marrow erythroblast-erythrocyte micronucleus test and the in vitro CHL/IU cell...
line micronucleus test.

Materials and Methods

Chemicals

InCl₃·4H₂O (InCl₃; 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 supplied by Sigma (MN, USA). NADH was obtained from WAKO Pure Chem (Osaka, Japan). S9 was supplied by Sigma (MN, USA). 5, 6-benzoflavone-induced male Sprague-Dawley rats were used for this study. A 1 ml aliquot of S9 mix containing 0.1 ml/5 S9, 8 µmol MgCl₂, 33 µmol KCl, 5 µmol glucose-6-phosphate, 4 µmol NADPH and 4 µmol NADH was used for the study.

In vivo micronucleus test

1. Experimental animals

BALB/c AnNCrlCrj 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 (Yokohama, Kanagawa, Japan). Significant increases in the micronucleus induction were statistically evaluated using the Cochran-Armitage test with Fisher’s exact test. The data were compared with those of the concurrent negative control by the Cochran-Armitage test with Fisher’s exact test.

Results

In vivo micronucleus test

The frequency of micronuclei induced by InCl₃ 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 InCl₃, 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

In vitro micronucleus test

1. Test chemical treatment and slide preparation

This study was performed as described by Schmid with minor modifications. In the continuous treatment method, cultures of 1×10⁶ CHL/IU cells were seeded in 60 mm plastic dishes and changed to fresh medium containing InCl₃ 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 InCl₃ 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 InCl₃ 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.

2. Microscopic 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.

In vitro micronucleus test

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In vivo micronucleus test

The mice were given water and a CRF-1 diet supplied by Charles River Japan (Yokohama, Kanagawa, Japan). They were maintained in Eagle’s minimum essential medium (medium) supplemented with 10% heat inactivated (56°C for 30 min) calf serum.

2. Rat 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 ml aliquot of S9 mix containing 0.1 ml/5 S9, 8 µmol MgCl₂, 33 µmol KCl, 5 µmol glucose-6-phosphate, 4 µmol NADPH and 4 µmol NADH was used for the study.

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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/ml 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/ml 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/ml 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/ml of InCl$_3$, after continuous treatment for 48 or 72 h compared with 0 mg/ml (Fig. 4–2).

The frequency of micronuclei increased dose-dependently ($p<0.01$) 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/ml of InCl$_3$ after 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/ml 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/ml 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/ml 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/ml) 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. $\square$, $\bigcirc$: $p>0.05$, $\blacksquare$, $\bullet$: $p<0.05$. 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/ml of InCl$_3$ after continuous treatment for 48 or 72 h, compared with 0 mg/ml.
InCl₃ 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/ml over 6–42 h and 4.69–18.75 µg/ml 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/ml) was 20.1 in the short time exposure method without S9 mix at a dose of 0.59 µg/ml 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/ml for 48 h) and in the short time exposure method with S9 mix (2.34 µg/ml for 6–42 h), respectively. The highest frequency of micronuclei induced by InCl₃ in all experiments was observed at doses of 18.75 µg/ml 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.

InP, which is a compound used in semiconductors, induces not only severe lung damage, but also cancer. Gottschling et al. 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. 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). There is a significant increase in micronucleated polychromatic erythrocytes in male, but not in female, mice exposed to InP. 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. 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
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. Lison et al. 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. The in vivo genotoxic potential of ITO particles for lung epithelial cells indicates their capacity to induce lung cancers. These results also show the relationship between free radicals and the genotoxic and/or tumorigenic potential.

Rowlands reported that indium compounds (including indium trichloride) show great potential as reagents in radical reactions. On the other hand, Dusre et al. 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. It is considered that the mechanism of micronucleus induction may therefore involve free radicals.

In the present study, InCl₃ 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 InCl₃ affects the bone marrow erythropoiesis by inducing chromosome aberration. Suzuki et al. 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 InCl₃ suppressed erythropoiesis division.

It is known that InCl₃ accumulates in bone marrow erythroblasts, thus making the compound useful in the diagnosis of myelopoiesis, and this results suggests that InCl₃ might induce micronuclei in erythroblasts.

A number of studies have shown that the administration of soluble indium as InCl₃, 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. The primary genotoxic mechanism of InCl₃ may thus involve oxidative stress, similar to InP and ITO. However, precancerous change in the lung...
was caused only by InAs when indium arsenide, gallium arsenic or arsenious acid were administered intratracheally to hamsters\textsuperscript{25}. These results also suggest that indium possibly induces carcinogenesis and mutagenesis in vivo.

Similarly, InCl\textsubscript{3} induced micronuclei in the \textit{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\textsubscript{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\textsubscript{3} is changed into In (OH)\textsubscript{3} in neutral and alkali solutions\textsuperscript{26}, In (OH)\textsubscript{3} may induce micronuclei under this experimental condition.

The growth of CHL/IU cells was inhibited dose- and time-dependently after treatment with InCl\textsubscript{3}. This is likely because the genotoxic effects of InCl\textsubscript{3} cause the micronucleus induction to occur in tandem with the extent of growth inhibition of the CHL/IU cells.

Based on the \textit{in vivo} and \textit{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\textsubscript{3} did not induce mutagenic activity with and without S9mix in the TA100, TA2637, TA94, TA98, WP2uvrA\textsuperscript{+} and WP2uvrA\textsuperscript{−} strains. These results suggest that InCl\textsubscript{3} may not induce mutagenicity in a bacterial mutation test system\textsuperscript{27}, however, it did induce micronuclei in mammalian cells in both \textit{in vivo} and \textit{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\textsubscript{3}.

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