Analysis of DNA Adducts after Exposure to 1,4-Dichlorobenzene by $^{32}$P-Postlabeling Technique

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To evaluate the genetic toxicity of 1,4-dichlorobenzene ($p$-DCB), DNA adducts of $p$-DCB were analyzed in in vitro and in vivo. In the in vitro study, calf thymus DNA (400 µg/ml) was mixed with $p$-DCB (100 µM), liver microsome (1 mg protein/ml) and an NADPH-regenerating system. After incubation at 37°C for 1 hr, DNA was isolated and purified for DNA adduct analysis. There were no DNA adducts detected when $p$-DCB was metabolized by rat, mouse or human liver microsomes. In the in vivo study, Fischer 344 rats were given different kinds of cytochrome P450 (CYP) inducers and then injected with $p$-DCB. Twenty-four hr after injection, livers were collected from the rats for DNA adducts analysis. We did not find any $p$-DCB DNA adducts in rat livers pre-treated with ethanol, phenobarbital or 3-methylcholanthrene, respectively. In conclusion, no $p$-DCB DNA adducts were found either in the in vitro or in vivo studies. In this study, we demonstrated that $p$-DCB is not genotoxic.

Key words —— 1,4-dichlorobenzene, DNA adduct, mutagenicity, genetic toxicity, P450

INTRODUCTION

The chemical 1,4-dichlorobenzene ($p$-DCB) is an important environmental pollutant that is ingested by the general population in Japan. This compound is a volatile organic solid used primarily as a space deodorant and moth repellent and is derived from insecticides for clothes or toilets, and is released to ambient air by volatilization. To date, it has been reported that $p$-DCB is not mutagenic in Salmonella typhimurium strains TA1535, TA1537, TA1538 or TA100. However, $p$-DCB showed mitogenic stimulation of hepatocellular proliferation in treated rodents. A link between cytotoxicity and binding of chlorobenzene to proteins has been postulated. $p$-DCB is not DNA reactive, as determined by mutagenicity, genotoxicity and clastogenicity assays, but some researchers injected $[U-^{14}C]$chlorobenzene into Wistar rats and BALB/c mice, then analyzed the isotope labeling of liver tissue, which indicated that chlorobenzene is capable of interacting with DNA. One study evaluated the unscheduled DNA synthesis and replicative DNA synthesis by treating rats with $p$-DCB. To examine whether $p$-DCB forms DNA adducts, we investigated the DNA adducts using the $^{32}$P-postlabeling technique, which allows us to detect and quantitate adducts at the level of one adduct per $10^{10}$ nucleotides. This technique is more sensitive than injecting isotope to animal body in detecting DNA adducts. In this paper, we report the non-genotoxicity of $p$-DCB in terms of binding to DNA in vivo and in vitro.

MATERIALS AND METHODS

Regarding animal treatment, 6-week-old male Fischer 344/NSlc rats, weighing 90–110 g, were obtained from Nippon SLC, Inc. (Hamamatsu, Japan). Animals were pre-treated with ethanol (10% in drinking water for more than 10 days), phenobarbital (80 mg/kg in 0.9% NaCl, i.p. injection, once a day for 3 days), 3-methylcholanthrene (25 mg/kg in olive oil, i.p. injection, once a day for 3 days), respectively, to induce different types of cytochrome P450. On the 4th day rats were given single i.p. injection of 300 or 600 mg/kg of 1,4-dichlorobenzene dissolved in olive oil. The control group was given olive oil alone and the positive control group was given single i.p. injection of BaP (100 mg/kg), 3 rats were treated in each group. Animals were killed 24 hr after injection. Liver was collected and stored at −80°C until DNA isolation. Microsome-mediated in vitro interaction Rat and mouse microsomes were prepared from male F344 rats and male BDF1 mice respectively. Animals were treated with phenobarbital, ethanol, dexamethasone and 3-methylcholanthrene. We analyzed with mixed microsome (microsomes from above 4 inducers were...
mixed with same volume). Human microsome was purchased from Gentest Corporation (Woburn, U.S.A.) p-DCB (100 µM) dissolved in dimethyl sulfoxide (DMSO) (final concentration < 2%) was incubated with human microsome and mixed microsome (1 mg protein/ml) from rat and mouse, calf thymus DNA (CT-DNA, 400 µg/ml), and a NADPH regenerating systems in 50 mM Tris–HCl buffer, pH7.4. After incubation for 1 hr in a shaking water bath at 37°C, the reaction was terminated by centrifugation (5000 rpm, 5 min) to remove the microsome proteins. Samples were extracted three times with water-saturated ethyl acetate to remove unreacted metabolites or the parent compound. The aqueous phase was briefly evaporated to remove ethyl acetate and the DNA was further purified by enzymatic treatment and solvent extractions.10)

**DNA Isolation** — DNA was extracted sequentially with phenol, phenol: CIAA (chloroform : isoamyl alcohol = 24 : 1) and CIAA, and recovered by ethanol precipitation. DNA concentration was determined spectrophotometrically.

**Analysis of DNA Adducts** — DNA adducts were analyzed by 32P-postlabeling after enhancement of assay sensitivity with nuclease P1. Briefly, DNA was digested with micrococal nuclease and spleen phosphodiesterase (enzyme : substrate, 1 : 5 w/w, 5 hr, 37°C). Adducts were enriched by nuclease P1 and labeled by T4 polynucleotide kinase catalyzed phosphorylation in the presence of a molar excess of [γ-32P]ATP. Labeled adducts were separated by multidirectional PEI-cellulose TLC using 1.0 M sodium phosphate, pH 6.0 for D1; 4.5 M lithium formate/8.5 M urea, pH 3.5 for D3; isopropanol : 4 M ammonium hydroxide (1 : 1) for D4 and 1.7 M sodium phosphate, pH 5.7 for D5. Adducts were detected with a Bio-Image Analyzer (FLA-3000; Fuji Photo Film Co. Ltd. Tokyo, Japan) after exposing the TLC sheet to the Fuji imaging plate.

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**RESULTS AND DISCUSSION**

In *in vitro* study, after incubation of p-DCB with calf thymus DNA in the presence of rat, mouse or human microsomes for 1 hr at 37°C, DNA adducts were analyzed by the 32P postlabeling method. No DNA adducts were detected after bioactivating with rat, mouse or human microsomes. BaP was set as a positive control here. We could detect 2 to 4 DNA-BaP adduct spots (Fig. 1). In pilot study, in order to compare the recovery of DNA adducts in the two commonly used enrichment procedures, adducts were enriched with both the nuclease P1 and n-butanol. There were no differences between the two enrichment methods.

In *in vivo* study, Male F344 rats were pre-treated with ethanol, phenobarbital, or 3-methylcholanthrene, respectively, to induce different types of CYP or untreated, and then given p-DCB (300, 600 mg/kg). We did not detect any DNA adducts in rat livers that were untreated or pre-treated with ethanol or phenobarbital (data not shown). Two spots of DNA adduct were detected in livers from 3-methylcholanthrene pre-treated rats. However, there was no difference found between the chromatography profiles of the groups treated with 3-methylcholanthrene alone and those treated with both 3-methylcholanthrene and p-dichlorobenzene. Comparing panel B with E, we concluded that the two spots, i.e., spot 1 and spot 2, were derived from 3-methylcholanthrene (Fig. 2 panel B). The spots, i.e., spot 3 and spot 4, were produced by BaP (Fig. 2 panel E).

To date, there are no data available regarding DNA adduct analysis of p-DCB. In this study, we suggest that p-DCB does not have any genotoxic potential. Our results are consistent with negative results in other mutagenicity or genotoxicity assays such as *Salmonella typhimurium* mutagenicity assay, the L5178Y/TK+/- mouse lymphoma assay, micronuclei assay and sister chromatid exchange assay.12)
Although p-DCB does not seem to be mutagenic in bacterial systems,\textsuperscript{13} this compound has been reported to be carcinogenic for rodents.\textsuperscript{14,15} However, this study showed that there were no DNA adducts formed in the male F344 rat liver after administration of p-DCB. Eldridge and colleagues observed induction of cell proliferation by p-DCB in the rat.\textsuperscript{3}\textsuperscript{)} In the literature, it was reported that hepatotumor formed after exposure to p-DCB and that the liver is more sensitive than other organs when chemicals are bioactivated or decomposed in liver.\textsuperscript{1}\textsuperscript{) Maybe DNA adducts can be detected in bronchia or lung in our future study. Since our study did not demonstrate any DNA adducts, the induction of cell proliferation by p-DCB is thought to play a key role in its hepatocarcinogenecity.

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REFERENCES


2) Shimizu, M., Yasui, Y. and Matsumoto N. (1983) Structural specificity of aromatic compounds with special reference to mutagenic activity in \textit{Salmonella typhimurium}—a series of chloro- or fluoro-


