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INTERNATIONAL ISOCYANATE INSTITUTE, INC.

201 Main Street, Suite 403 • La Crosse, WI 54601 • 608/796-0880 • FAX 608/796-0882

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Dear Sir/Madam:

The following information is being submitted by the International Isocyanate Institute (III) on behalf of its members pursuant to current guidance issued by EPA indicating EPA's interpretation of Section 8 (e) of the Toxic Substance Control Act. Neither III nor any member of III has made a determination as to whether a significant risk of injury to health or the environment is actually presented by the findings.

The enclosed abstract recently came to the attention of the members of III. In the abstract, the authors, Zhong and Siegel, concluded that their study indicated that MDI aerosol exposure of Brown-Norway rats cause a dose dependent increase in the frequency of micronucleated polychromatic erythrocytes. The underlying mechanism for this observation may be non-genotoxic, although the mode of action has not been clearly established.

Sincerely,

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Induction of Micronuclei Following Exposure to Methylene Di-phenyl Diisocyanate:

Potential Genotoxic Metallo-lites

Zhong, B.-Z. and Siegel, P. D.

**This work has been submitted
for possible publication.**

DRAFT

Health Effect Laboratory Division, National Institute for Occupational Safety and Health.

Morgantown, WV 26505-2888

Address correspondence to:

Dr. Paul D. Siegel

Analytical Services Branch, M/S H117

HELD, NIOSH

1095 Willowdale Road

Morgantown, WV 26505-2888

E-mail: pds3@cdc.gov

Telephone: (304) 285-5855

Fax: (304)285-6321

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Induction of Micronuclei Following Exposure to Methylene Di-phenyl Diisocyanate:

Potential Genotoxic Metabolites. Zhong, B.-Z. and Siegel, P. D. (1999) *Toxicol. Sci.*

Methylene di-phenyl diisocyanate (MDI) is used to make polyurethane products. The predominant occupational disease attributed to diisocyanates, including MDI, is asthma; however, the potential for genotoxicity has also been of concern. Diisocyanates are very reactive compounds that can undergo nonenzymatic hydrolysis to form methylenedianiline (MDA), or react under physiological conditions with primary amines to form ureas and/or with thiols to form labile thiol acid esters. MDA is a carcinogen in animals and a suspected carcinogen in humans. Brown Norway rats (BNR) were exposed to either 7 or 113 mg/m³ MDI aerosol for 1 hr, 1 x/week for 3 weeks and sacrificed 1 week later. Micronuclei (MN) formation was assessed from bone marrow polychromatic erythrocytes (PCE). Dose dependent increase in the frequency of micronucleated polychromatic erythrocytes (MNPCE) was noted. *In vitro* exposure of Chinese hamster lung fibroblasts (V79) to MDA, or MDI-thiol acid esters, but not MDI, significantly increased the frequency of MN. MDI-thiol acid ester exposed cell cultures did not have detectable levels of MDA. A significant increase in the number of V79 cells in metaphase, as well as, cells with a precipitant within both the cytoplasm and nuclei were noted in MDI-glutathione exposed cultures. The results of this study indicate that MDI aerosol exposure can cause MN formation through either the hydrolysis of MDI to MDA or the formation of thiol acid esters.

Key Words: Micronucleus; Methylene Di-phenyl Diisocyanate; Metabolites;

Brown Norway Rats; V79 cells

Introduction

Methylene di-phenyl diisocyanate (MDI) is an important industrial chemical used to make polyurethane products such as foams, wood binders, and polyurethane elastomer casting systems. The occupational exposure to MDI may occur in a number of occupations including adhesive and isocyanate resin workers, organic chemical synthesizers, paint sprayers, rubber polyurethane workers, ship burners, textile processors and wire coating workers (USEPA, 1984). Exposure occurs mainly by the dermal and inhalation routes. The major hazards associated with MDI are immediate-type pulmonary hypersensitivity reactions and direct irritant toxic responses. The asthmatic potential of diisocyanates has been known since 1951 (Fuchs *et al.*).

Genotoxic potential of aromatic diisocyanates has also been a concern, but very few studies pertaining to this area can be found. Marczynski *et al.* (1992) reported DNA damage in leucocytes of a worker following MDI inhalation challenge. Carcinogenicity studies of MDI exposure to rats has been negative, but an increase in pulmonary adenomas was noted (Reuzel *et al.*, 1994). There has been no reported studies examining the induction of MN following inhalation exposure to MDI.

A potential hazard from MDI exposure may result from water hydrolysis to methylenedianiline (MDA). MDA induced hepatotoxicity in rats (Baillie *et al.*, 1993). MDA is mutagenic in a number of assays for genotoxicity including: mutation in salmonella microsomal assay (Andersen *et al.*, 1980; Woolrich, 1982; Tanaka *et al.*, 1985; Cocker *et al.*, 1986), strand break and unscheduled DNA synthesis in hepatocytes (Swenberg, 1981; Parodi *et al.*, 1981; Mori *et al.*, 1988), and chromosomal aberrations and sister chromatid exchanges in human lymphocytes (Mäki-

paakkanen et al., 1987; Goswami, 1986; Kligerman *et al.*, 1987). The results pertaining to MDA induction of MN formation were inconsistent (USEPA, 1984; Shelby *et al.*, 1987), MDA has been classified as a carcinogen in animals and is a suspected carcinogen in human (IARC, 1986).

Another aromatic diisocyanate, toluene diisocyanate (TDI), did not produce a carcinogenic effect in rats or mice chronically exposed to up to 0.15 ppm TDI (Loeser, 1983). This study, however, used low exposure levels versus maximum tolerated dose. The dose used may have been insufficient to discern a carcinogenic response (NTP, 1986) A National Toxicology Program report indicates that TDI is a carcinogen in rats and mice when administered by gavage (NTP, 1986).

Many products can be formed *in vivo* by exposure to MDI, due to its great reactivity. Hydrolysis to MDA is slow in comparison to its reaction with thiols and amines under physiological conditions. Reaction kinetics favor conjugation to thiols > amines. Diisocyanates can also react with hydroxy groups in hydrophobic regions of proteins (Kennedy and Brown, 1992). The reaction to thiols to form thiol acid esters is of particular interest. These compounds are labile under physiological pH potentially regenerating free diisocyanate at sites distal to the lung. The present study documents that exposure to aerosolized MDI produces genotoxicity in rats, and attempts to identify potential MDI genotoxic metabolites using *in vitro* MDI, MDA, and MDI-cysteine and MDI-glutathione thiol acid ester exposures to Chinese hamster lung fibroblasts.

Materials and Methods

Chemicals

4,4'-MDI, acetone, absolute methanol and chromatographic solvents were purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) and Giemsa stain, 4,4'-MDA, L-cysteine, glutathione were obtained from Sigma company (St. Louis, MO) May-Grünwald stain was from Harleco (Gibbstown, NJ) and Diff Quik was purchased from Baxter Scientific (McGaw, IL). Dimethyl sulfoxide (DMSO) was from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Animals

Male Brown Norway rats (BNR) from Harlan Laboratory (Indianapolis, IN), 9-10 weeks old with a mean body weight of 200g were used. The rats were housed in groups of two per cage and acclimatized for 1 week before exposure. Water and Purina laboratory rodent chow were provided *ad libitum*.

Cell Line and Culture Conditions

The Chinese hamster lung fibroblasts (V79) cell line were kindly supplied by C.C.Chang (Michigan State University, East Lansing, MI). Cells were subcultured every 3-4 days and maintained as a monolayer in a 75 cm² tissue culture flask (Corning Costar Corporation, Cambridge, MA) with 15 ml complete medium consisting of 90% Minimum Essential Medium (MEM; Sigma, ST. Louis, MO), 10% fetal bovine serum (FBS, Sigma), 2mM L-glutamine (Sigma), 100 units penicillin/ml, and

100 μ g streptomycin/ml (Sigma). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ for all experiments. Cell cultures used for exposures did not exceed the 17th passage.

Inhalation exposure

Rats were exposed in a 15 liter-whole body plastic inhalation chamber. MDI condensation aerosols were generated by bubbling 4 l/min or 1 l/min of dry, clean air through an impinger containing 4 gm. of MDI monomer heated to 125°C. The condensate was diluted with filtered room air to provide a total flow of 8 l/min through the chamber. MDI aerosol chamber concentrations were determined by gravimetric analysis. Air was sampled at 1 l/min through a 37 mm PTFE 2 μ m pore filter. The mass median aerodynamic diameter of the aerosol particles was 0.8 μ m with a σ_g of 0.8. Chemical analysis of MDI chamber concentrations were assessed in preliminary studies by extracting the filters with dry acetone and measuring the MDI content using both a modified Marcallei method (Rando and Hammad, 1989) and tryptamine derivitization (Wu *et al.*, 1990) isocyanate methods.

Multiple exposure groups of 6 rats were exposed to MDI aerosol at the concentration of 113.2 \pm 24.2 SD (High) or 7.05 \pm 4.3 SD (Low) mg/m³ for 1 hour a day, 1 day per week for a total of 3 weeks. Rats were exposed 2 at a time in the inhalation chamber. Rats were anesthetized with a dose of 50 mg/kg of pentobarbital by intraperitoneal injection and sacrificed one week after the last exposure. The bone marrow from each animal was harvested from the femur. A control group of 4 rats was used concurrently and bone marrow cells were obtain for analysis.

Sample preparation and in vitro exposure assay

MDI was weighed and melted at temperature 90°C in a glass tube, diluted with dry DMSO to make a stock suspension at a concentration of 100, 50, 25, 10 mg/ml respectively. MDA was dissolved in dry DMSO directly to make a stock solution and diluted with dry DMSO to the concentrations of 50, 25, 10, 5.0 mg/ml respectively. MDI-cysteine or MDI-glutathione conjugates were prepared immediately prior to use by slowly dripping 0.3 mM MDI/dry acetone (2 ml) into a 10 ml solution of 0.6 mM cysteine or glutathione in acetonitrile/water (7:3) while stirring over a 30 min period. The resultant precipitants were recovered by filtration, rinsed with dry methanol to remove any MDA that may have formed and then with dry acetone. The MDI-cysteine and MDI-glutathione conjugates were suspended in dry DMSO at 100, 50, 25, 10, 5.0 mg/ml, and 50 µl was added to the medium for exposure. The S9 fraction of microsomes was made from the livers of Aroclor-1254-treated male Sprague Dawley rats. A 10 % concentration (w/v) of S9 mixture was prepared in a buffer containing 0.1 M NADP, 1.0 M glucose-6-PO₄, 0.4 M MgCl₂, 1.65 M KCl, 0.2 M PO₄ and added to the exposure medium to assess if metabolic activation was required. Approximately 6 x 10⁵ cells were seeded in a 25 cm² tissue culture flask (Corning Costar Corporation, Cambridge, MA) with 5 ml Minimum Essential Medium (MEM, Gibco) for 24 h. Medium was replaced with 5 ml of phosphate buffer solution (PBS, pH=7.2) and 50 µl of MDI, MDA, MDI-cysteine or MDI-glutathione were added to obtain final concentrations ranging from 50 to 1000 µg/ml. Cultures were challenged for 5 h. DMSO controls were run concurrently for each experiment. The treatment levels were selected for each chemical to allow >50 % survival relative to control cells. Twenty percent (v/v) of the S9 buffer solution was added to the medium for samples

to assess role of metabolic activation. Duplicate exposures were made for each concentration, and 2 experiments were performed.

Micronuclei assay

Preparation from bone marrow cells: Bone marrow cells were isolated from the both sides of the femurs by cutting the proximal end of the bones. Cells were flushed from the bone cavity into a centrifuge tube with 5 ml FBS solution, gently pipetted and debris was removed to produce a fine cell suspension. Slides were made by dropping 1-2 drops of cell suspension onto each pre-cleaned slide and a smear was made by drawing the beveled edge of another glass slide across at 45 degree angle. The slides were allowed to air-dry overnight and then fixed in absolute methanol for 10 min. Air-dried slides were stained sequentially with (1) 0.5% of May Grünwald stain in absolute methanol for 2 min, (2) then May Grünwald:distilled water (1:1) for 2 min, (3) and Giemsa:distilled water (1:6) for 8 min. The slides were rinsed with distilled water and left to air-dry. The mature erythrocytes (NCEs) showed a intense, pink red color and the polychromatic erythrocytes (PCE, pre-mature erythrocytes) displayed a strong, bluish tint. All slides were coded, and scored under 1000X magnification by a single scorer. The proportion of PCEs among 1000 erythrocytes was determined and the ratio of PCEs to NCEs to indicate bone marrow toxicity and they were expressed as a group average ratio in the experiments. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) for each animal per concentration was based on 5000 PCEs per each slide and 2 slides per group were scored. The criteria used to score MN followed that reported by Schmid (1976). Statistical analysis was carried out by the trend test (Margolin et al., 1986). Linear regression analysis was also used to evaluate the concentration-response relationship. The

significance between different treatment groups and control group was analyzed by means of chi-square test. The grouped *t* test for multiple samples was used to compare FCEs between treated and control animals (MacGreger *et al.*, 1987).

Preparation from V79 cells: The MN assay in V79 was used according to the procedure of Zhong *et al.* (1997). The cells were harvested after 24 h post-incubation by trypsinization, rinsed with PBS, centrifuged and resuspended in 0.5 ml PBS. Slides were made using cytospin (Shandon, Pittsburgh, PA) and stain with DiffQuik stain. The frequency of MN was based on 4000 cells scored (1000 cells/slide x 4) per group. The average number of cells with MN per 1000 V79 cells \pm standard deviation were expressed. Statistic analysis was performed on the trend and chi-square tests.

Chemical Analysis of MDI-Conjugates, and MDA

Isocyanate thiol acid esters are acid stable and reversible at neutral or alkaline pH forming free isocyanate. Suspensions of isocyanate thiol conjugates were made in 0.01 M HCl and 0.01 M NaOH. Release of free MDI was evaluated by measuring formation of the hydrolysis product MDA by GC-MS (Hewlett Packard, Pittsburgh, PA). The MDI-glutathione and MDI-cysteine conjugates were found to be acid stable and alkaline labile. This is consistent with the presence of isocyanate thiol acid ester products. The potential for formation of MDA in cell culture exposed to MDI thiol acid esters was assessed using a HPLC-electrochemical method similar to that reported by Robert *et al.* (1995). The HPLC system consisted of a SIL 6B autoinjector, two LC600 pumps, a SPD 6AV spectrophotometric detector, a LPI-6B interface, Class VP software system (Schimadzu Scientific Instruments, Inc., Columbia, MD) and a Coulochem II electrochemical detector (ESA, Bedford, MA). The samples (10 μ l) were injected onto a C18, 250 x 4.6 mm, 5 μ m pore Luna

column (Whatman Inc., Clifton, NJ) . The sample was eluted from the column at 1 ml/min using a mobile phase of 50/50, acetonitrile/ 0.1M acetate buffer, pH 5.5. MDA was measured using an absorbance of 275nm, and 900mV on the electrochemical detector.

Results

The results of MN induction in rat bone marrow erythrocytes after MDI inhalation are shown in Table 1. MN formation was significantly induced in PCEs compare to the control following exposure to MDI aerosol at concentrations of 7.1 or 113.2 mg/m³, $p < 0.05$, $p < 0.01$ respectively. The Z value was 11.07 in the trend test ($p = 0.01$), and the correlation coefficient for linear regression analysis was 0.99. The number of PCEs with MN increased in the bone marrow in a MDI exposure concentration-dependent manner. No difference was found in the ratio of PCEs and NCEs between exposure and control groups. Figure 1 is a photomicrograph of bone marrow cells from a MDI exposed Brown Norway Rat. The arrow is pointing to a MNPCE.

The frequency of MN increased following *in vitro* treatment of V79 cells with MDA, MDI-cysteine or MDI-gluthathione, but not with MDI (Table 2, Fig 2a). Metabolic activation using the S9 microsomal fraction significantly increased MDA induced MN formation at only the highest (500 $\mu\text{g/ml}$) exposure concentration. The V79 cells treated with MDI conjugates, with or without S9 activation (S9 data not shown), displayed similar MN induction at a concentration range of 50 to 1000 $\mu\text{g/ml}$. A significant concentration-related increase in frequencies of MN was observed in cultures treated with MDI-glutathione. However, in the MDI-cysteine exposure group a dose-response relationship was not evident. This may be attributed to cytotoxicity of the conjugate at higher concentrations.

All cultures were quantitatively assayed for the presence of MDA using HPLC-EC. MDA was not detected in the culture medium supernatant or sediment from V79 cell cultures exposed to MDI only, MDI-cysteine, or MDI-glutathione conjugates, with or without S9. MDA was

quantified both in the supernatant and sediment of the culture medium from V79 cells exposed to MDA. Approximately, 20% of the MDA was found in the supernatant and 80% in the sediment from each exposure concentration.

The MDI-glutathione thiol acid ester produced an increase number of V79 cell in metaphase (Table 3). A precipitant was noted in both the cytoplasm and nuclei of many of the cells. These effects were dose-dependent up through 500 $\mu\text{g/ml}$ MDI-GSH. The number of binucleated cells was not significantly altered by treatment. Figures 2b, c are photomicrographs of MDI-GSH cultures demonstrating cells apparently arrested in metaphase, the presence of intracellular precipitants and MN. The localization of the precipitants in both the cytoplasm and nuclei was confirmed using confocal microscopy (Fig 2d).

Discussion

MDI is a highly reactive, electrophilic compound which can covalently bind to proteins forming macromolecular adducts. Its reactivity toward DNA is still of issue. MDI-DNA adducts were found only in the olfactory epithelium and not in other respiratory tissue or peripheral organs after inhalation exposure of rats to MDI (Vock *et al.*, 1996). The DNA adduct detected co-chromatographed with that formed in the liver following ingestion of the MDI hydrolysis product, MDA. Vock and Lutz (1997) found equivocal results of DNA adducts when MDI was applied topically to rats. TDI-DNA, but not MDI-DNA adducts could be found when the respective isocyanate was incubated with DNA, *in vitro* (Peel *et al.*, 1997).

In recent years determination of MDA in hydrolyzed urine or plasma have been suggested for biological monitoring of MDI exposure in human or experimental animals (Brunmark *et al.*, 1995; Sepai *et al.*, 1995). MDA has two aniline rings linked by a methylene bridge and is structurally related to polycyclic aromatic carcinogenic compounds such as benzidine and aminobiphenyl (International Agency for Research on Cancer, 1971). MDA is a known carcinogen in a number of animal studies (Andersen *et al.*, 1980; Woolrich, 1982; Tanaka *et al.*, 1985; Cocker *et al.*, 1986). *In Situ* hydrolysis of MDI to MDA is a potential mechanism of DNA adduct formation following MDI exposure.

The formation of MN is a consequence of chromosomal breakage and/or spindle-fiber dysfunction induced by clastogens and aneuploidogens. They are small, contain chromatin and are not incorporated into daughter nuclei following mitosis. Hence, the MN assay can be used to predict structural or numerical chromosomal aberrations. MN formation in bone marrow polychromatic

erythrocytes has often been used as a genetic endpoint. The results of the present study showed that the MN were induced in bone marrow polychromatic erythrocytes in a dose-related manner, after exposure of rats to 7.1 and 113.2 mg/m³ MDI. The ratio of NCEs/PCEs was not different between control and exposure groups suggesting that there was no observable bone marrow cytotoxicity. At least two potential mechanisms exist that may be responsible for the MN results observed in the studies. Hydrolysis and decarboxylation of MDI to MDA, with subsequent enzymatic activation in the liver to a DNA-relative intermediate could produce MN in the bone marrow. An enzyme-independent reaction of nucleophiles with the electrophilic isocyanate (NCO) group also may exist. The NCO group is very reactive and would need to cross several barriers intact to react with DNA or DNA associated proteins. Reaction of NCO to thiols produces a reversible bond that may possibly protect the isocyanate group and allow entry into the cell and the nuclear compartment. Once inside the cell the thiol acid ester may undergo hydrolysis producing a sulfur to nitrogen, or sulfur to oxygen exchange resulting in MDI conjugated intracellular constituents.

V79 Chinese hamster fibroblast cell cultures were employed to explore the potential mechanisms of MN formation induced following MDI exposure. The cells were challenged for 5 h., individually, *in vitro* with MDI, MDA, MDI-cysteine, and MDI-glutathione. MN was not induced, with or without S9 medium, in V79 cells treated with fresh MDI at a concentration of 100-1000 μ g/ml. MDA was not detected using HPLC-EC analysis in the supernatant or sediment. Exposure of the cells to MDA induced MN, with or without S9, in a dose-dependent manner at a concentration range of 50-500 μ g/ml. MDA was detected in the supernatant and sediments with 20% and 80% in each respective fraction. The increased MN frequency with addition of S9 at only the highest MDA exposure suggest that the V79 cells may contain metabolic enzymes capable of

activating MDA. Similar dose-dependent induction of MN following incubation of the cells with MDI-cysteine and MDI-glutathione was observed. MDA was not detected from these cultures indicating that the mechanism of MN induction was not via hydrolysis of MDI to MDA. It is not known if the MDI-thiol acid esters effect was via DNA, or protein adduct formation and possibly reaction with spindle components. The observed intracellular and intranuclear precipitants, and increased percent of cells in mitosis following MDI-GSH exposure suggest that the mechanism may involve the reaction of MDI with spindle components.

It should be noted that the Brown Norway rat model may be of particular utility for the study of diisocyanate toxicology. This model has been used in our laboratory because of its tendency to produce high levels of IgE. The bone marrow from rat models of MN genotoxicity test have severe interference caused by the great numbers of basophilic mast cell granules that are usually difficult to distinguish from the MN, especial the Fischer-344 strain rat (MacGregor *et al.*, 1987). The Brown Norway rat's bone marrow contained very few of these mast granules and did not require purification (Fig. 1). The relative sensitivity to genotoxic agents of this model vs others that have been used for such studies is not known.

The results observed in this study may have direct relevance to the mechanism of NCO induced asthmas. Maestrelli *et al.* (1994) reported that TDI exposure produced a CD8⁺ T-cell reaction in the airways, implicating a role of these cells in NCO asthma. This would require NCO-hapten processing by the endogenous (intracellular) pathway for presentation of the antigen MHC class I molecules. Conjugation of NCO to GSH may protect the NCO group from reaction to extracellular amines or hydrolysis and allow uptake into the antigen processing cells through a peptide or GSH transport molecule. Once inside the cell NCO may be slowly released, react with

intracellular proteins and be processed through the endogenous pathway.

In summary, the present research has demonstrated that inhalation of MDI aerosol produces dose-dependent genetic toxicity in the Brown Norway rat. Both MDA and MDI-thiol acid esters have been shown to be potential genotoxic metabolites of MDI. Future studies will be aimed at delineation of the mechanism of genetic damage observed.

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