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Induction of Micronuclei following Exposure to Methylene Di-phenyl Diisocyanate: Potential Genotoxic Metabolites

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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/m^3 MDI aerosol for 1 h/week ×3 weeks and sacrificed 1 week later. Micronuclei (MN) formation was assessed from bone marrow polychromatic erythrocytes (PCE). A dose-dependent increase in the frequency of micronucleated polychromatic erythrocytes (MN-PCEs) was noted. *In vitro* exposure of Chinese hamster lung fibroblasts (V79) to MDA or MDI-thiol conjugates, but not to MDI, significantly increased the frequency of MN. MDI-thiol conjugate-exposed cell cultures did not have detectable levels of MDA. A significant increase in the number of V79 cells in metaphase, as well as the number of cells with precipitants 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 possibly the formation of thiol conjugates.

Key Words: **micronucleus; methylene di-phenyl diisocyanate; metabolites; Brown Norway rats; V79 cells.**

MDI is an important industrial chemical used to make polyurethane products such as foams, wood binders, and polyurethane elastomer-casting systems. 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 (U.S. EPA, 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 asthmatogenic potential of diisocyanates has been known since 1951 (Fuchs and Valade, 1951).

The 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. DNA double-strand breaks were induced in cultured human lung epithelial cells (A549) (Vock, *et al*., 1998). Carcinogenicity studies of MDI exposure to rats has been negative, but an increase in pulmonary adenomas was noted (Reuzel *et al*., 1994). There have 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 induces hepatoxicity in rats (Bailie *et al.*, 1993). MDA is mutagenic in a number of assays for genotoxicity, including mutation in the *Salmonella* microsomal assay (Andersen *et al.*, 1980; Cocker *et al.*, 1986; Tanaka *et al.*, 1985; Woolrich, 1982), strand break and unscheduled DNA synthesis in hepatocytes (Mori *et al.*, 1988; Parodi *et al.*, 1981; Swenberg, 1981), and chromosomal aberrations and sister chromatid exchanges in human lymphocytes (Goswami, 1986; Kligerman *et al.*, 1987; Mäki-paakkanen *et al*., 1987). The results pertaining to MDA induction of MN formation were inconsistent (Shelby *et al.*, 1987; U.S. EPA, 1984). The results from exposure of rats and mice to MDA in drinking water for 103 weeks showed the carcinogenic effects (National Toxicology Program, 1984). MDA has been classified as a carcinogen in animals and is a suspected carcinogen in humans (International Agency for Research on Cancer, 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 the maximum tolerated dose. The dose used may have been insufficient to discern a carcinogenic response; a National Toxicology Program report indicates that TDI is a carcinogen in rats and mice when administered by gavage (National Toxicology Program, 1986).

Many products can be formed *in vivo* by exposure to MDI, due to its great reactivity. Hydrolysis to MDA is slow in

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comparison to its reaction with thiols and amines under physiological conditions. Reaction kinetics favor conjugation to thiols > amines. Diisocyanates can also react with hydroxyl 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 we attempt to identify potential MDI genotoxic metabolites, using *in vitro* MDI, MDA, and MDI-cysteine and MDI-glutathione conjugate exposures of Chinese hamster lung fibroblasts.

MATERIALS AND METHODS

Chemicals. 4,4′-Methylene di-phenyl diisocyanate (MDI, CAS 101–68–8, $C_{15}H_{10}N_2O_2$), acetone, absolute methanol, and chromatographic solvents were purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS), Giemsa stain, methylenedianiline (MDA, CAS 101–77–9, $C_{13}H_{14}N_2$), L-cysteine, and 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) were used. The rats, 9–10 weeks old and with a mean body weight of 200 g, were housed in groups of 2 per cage and acclimatized for 1 week before exposure. Water and Purina laboratory rodent chow were provided *ad libitum*.

Cell line and culture conditions. C. C. Chang (Michigan State University, East East Lansing, MI) kindly supplied the Chinese hamster lung fibroblast (V79) cell line. Cells were subcultured every 3–4 days and maintained as a monolayer in a 75 cm^2 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), 2 mM L-glutamine (Sigma), 100 units penicillin/ml, and 100 μg streptomycin/ml (Sigma). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for all experiments.

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 g 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 PFTE 2-μm pore filter. The mass median aerodynamic diameter of the aerosol particles was 0.8 μm 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 (Rando and Hammad, 1985) and tryptamine derivitization (Wu *et al.*, 1990) isocyanate methods.

Multiple exposure groups of 6 male rats were exposed to MDI aerosol at a concentration of 113.2 ± 24.2 SD (high) or 7.05 ± 4.3 SD (low) mg/m^3 for 1 h a day, 1 day per week for a total of 3 weeks. Rats were exposed, 2 at a time, in the inhalation chamber, then one week after the last exposure they were anesthetized with a dose of 50 mg/kg of sodium pentobarbital by intraperitoneal injection, and were sacrificed. The bone marrow from each animal was harvested from the femurs. A control group of 4 rats was used concurrently and bone-marrow cells were obtained for analysis. Genotoxic assessment was part of a larger study of MDI-induced immunological and inflammatory changes. The study plan employed was designed to optimize the immunological and inflammatory changes.

Sample preparation and* in vitro *exposure assay. MDI was weighed and melted at 90°C in a glass tube and then diluted with dry DMSO to make stock suspensions of 100, 50, 25, and 10 mg/ml. MDA was dissolved in dry DMSO at concentrations of 50, 25, 10, and 5 mg/ml. 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, or 5 mg/ml. Fifty μl of each stock suspension or solution was added to the medium for exposure.

In order to assess whether metabolic activation was required, an 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-PO_4, 0.4 M $MgCl_2$, 1.65 M KCl, and 0.2 M PO_4 and added to the exposure medium. Approximately 6×10^5 cells were seeded in a 25 cm^2 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 medium for samples to assess metabolic activation. Duplicate exposures were made for each concentration, and 2 experiments were performed.

Micronucleus Assay

Preparation from bone marrow cells. Bone marrow cells were isolated from both 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 a 45° 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% 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 normochromatic erythrocytes (NCEs) showed an intense pink-red color and the polychromatic erythrocytes (PCEs, pre-mature erythrocytes) displayed a strong bluish tint. All slides were coded and scored under 1000× magnification by a single scorer. The proportion of PCEs among 1000 erythrocytes was determined and the ratio of PCEs to NCEs was used to indicate bone marrow toxicity. The latter was expressed as a group average ratio in the experiments. The frequency of micronucleated polychromatic erythrocytes (MN-PCEs) for each animal per concentration was based on 5000 PCEs per slide, and 2 slides per animal were scored. The criteria used to score micronuclei (MN) followed that reported by Schmid (1976). Additional slides were stained with 0.02 mg/ml acridine orange in 0.2 M sodium phosphate buffer, pH 7.2 to 7.5 to verify that mast cell granules were not interfering with MN enumeration. Statistical analysis was carried out by the trend test (Margolin *et al.*, 1986). The significance between different treatment groups and the control group was analyzed by means of a Chi-square test. The grouped *t*-test for multiple samples was used to compare MN-PCEs between treated and control animals (MacGregor *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 a cytospin (Shandon, Pittsburgh, PA) and stained with Diff Quik stain. The frequency of MN was based

TABLE 1
Induction of Micronuclei in Rat Bone Marrow Polychromatic Erythrocytes following MDI Exposure

Group[a]	Concentration (mg/m^3)[b]	PCEs/1000 erythrocytes[b]	PCE/NCE ratio	Frequency of MN (%)[b,c]
Control	0.0	467.0 ± 22.5	0.88	0.95 ± 0.14
Low	7.1 ± 4.3	481.0 ± 6.7	0.93	1.50 ± 0.34*
High	113.2 ± 24.2	472.0 ± 18.4	0.89	4.10 ± 0.70**

[a]Six animals per group.
[b]Mean ± SD.
[c]10,000 PCEs were scored on 2 slides/animal.
*$p < 0.05$, ** $p < 0.01$ compared to control.

on 4000 cells scored (1000 cells/slide × 4) per group. The average number of cells with MN per 1000 V79 cells ± standard deviation were expressed. Statistic analysis was performed using the trend and Chi-square tests.

Chemical analysis of MDI-conjugates and MDA. Isocyanate thiol acid esters are acid-stable and reversibly form free isocyanate at neutral or alkaline pH. 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 an HPLC-electrochemical method similar to that reported by Robert *et al.* (1995). The HPLC system consisted of a SIL6B autoinjector, 2 LC600 pumps, an SPD 6AV spectrophotometric detector, an 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 μl) were injected onto a C18, 250 × 4.6 mm, 5 μm pore Luna column (Whatman, Inc., Clifton, NJ). Each sample was eluted from the column at 1 ml/min using a mobile phase of 50/50, acetonitrile/0.1 M acetate buffer (pH 5.5). MDA was measured using an absorbance of 275 nM, and 900 mV 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 compared to the control following exposure to MDI aerosol at concentrations of 7.1 and 113.2 mg/m^3; $p < 0.05$, $p < 0.01$, respectively. The Z value was 11.07 in the trend test ($p = 0.01$). The number of PCEs with MN increased in the bone marrow in an MDI exposure concentration-dependent manner. No difference was found in the ratio of PCEs and NCEs between exposure and control groups. Figure 1, left and right panels, are photomicrographs of bone marrow cells from an MDI-exposed Brown Norway rat. Each arrow points to an MN-PCE. Figure 1 also illustrates the absence of interference from mast cell granules.

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. 2 [upper left panel]). Metabolic activation using the S9 microsomal fraction significantly increased MDA-induced MN formation over that observed in the corresponding S9 microsome free cultures at only the highest (500 μg/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 μg/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 ex-

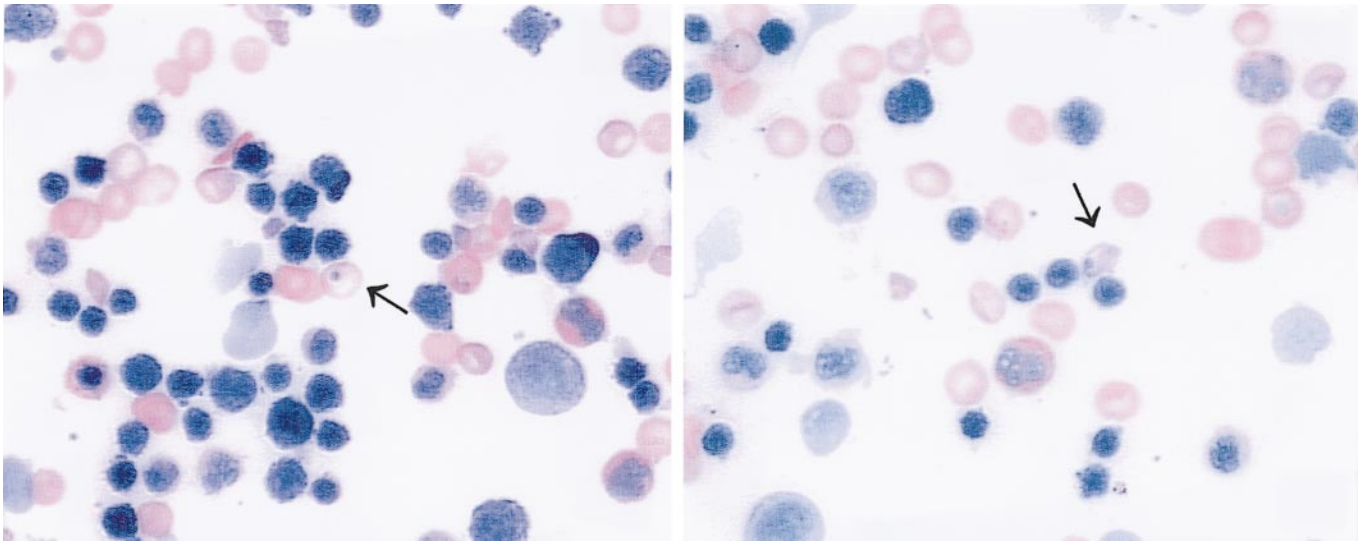

FIG. 1. MN-PCEs from bone marrow of BNR exposed to MDI. The photomicrographs demonstrate the absence of interference from mast cell granules. Magnification ×700. Right and left panel, arrows point to an MN-PCE.

TABLE 2
Frequency of Micronucleus Induction following Treatment with MDI or MDI Conjugates in V79 Cells

Group	MDI		MDA		MDI-cysteine	MDI-GSH
	No S9	With S9	No S9	With S9	No S9	No S9
Control	6.0 ± 0.8	7.0 ± 0.5	7.8 ± 0.9	7.0 ± 0.8	6.0 ± 0.0	6.0 ± 0.0
50 μg/ml	ND	ND	10.7 ± 2.2*	8.3 ± 1.7	15.3 ± 2.2**	ND
100 μg/ml	5.3 ± 1.9	5.5 ± 0.6	9.5 ± 2.4	11.0 ± 1.8*	17.0 ± 1.2**	10.3 ± 9**
250 μg/ml	6.5 ± 0.6	6.0 ± 0.0	18.0 ± 2.0**	16.0 ± 5.1**	10.3 ± 1.7*	13.0 ± 0.8**
500 μg/ml	5.0 ± 0.8	6.0 ± 0.8	29.7 ± 4.5**	43.7 ± 3.3**[a]	9.7 ± 1.9*	14.2 ± 2.9*
1000 μg/ml	6.7 ± 2.0	7.7 ± 2.6	ND	ND	ND	19.2 ± 1.5**

Note. Number of micronucleated cells/1000 cells (0 ± SD); 4000 cells scored/group; ND, not done.
[a] $p < 0.01$ compared to the corresponding culture without S9 microsomes.
*$p < 0.05$, **$p < 0.01$ compared to control.

posed 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 conjugate produced an increase in number of V79 cells in metaphase (Table 3). A precipitant was noted in both the cytoplasm and nuclei of many of the cells. Precipitants were clearly distinguished from MN by both staining characteristics and morphology. These effects were dose-dependent up through 500 μg/ml MDI-GSH. The nuclear division index was not significantly altered by treatment. The upper right and center left panels of Figure 2 are photomicrographs of an MDI-GSH culture demonstrating V79 fibroblasts with intracellular and intranuclear precipitants (arrows). Figure 2 (center right panel) is a photomicrograph of an MDI-GSH culture demonstrating cells in metaphase with the presence of intracellular precipitants (arrows). The localization of the precipitants in both the cytoplasm and nuclei was confirmed using confocal microscopy (Fig. 2, bottom panel, arrows).

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-eluted from the chromatographic column with that formed in the liver following ingestion of the MDI hydrolysis product, MDA. Vock and Lutz (1997) found equivocal DNA adduct results 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 has been suggested for biological monitoring of MDI exposure in human or experimental animals (Brunmark *et al.*, 1995; Sepai *et al.*, 1995). MDA has 2 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; Cocker *et al.*, 1986; Tanaka *et al.*, 1985; Woolrich, 1982). *in situ* hydrolysis of MDI to MDA is a potential mechanism of DNA adduct formation following MDI exposure. MDI can also react in the body with functional groups on biological molecules. Sepai *et al.* (1995) reported a number of MDI metabolites in rats, including hemoglobin adducts in the blood, and MDA and N-acetyl-MDA in hydrolyzed urine. There was no reduction of the MDI-hemoglobin adduct in the blood 7 days following an acute MDI exposure. MDA and N-acetyl-MDA were at measurable levels in the urine after 7 days, suggesting that at least some MDI metabolites are very persistent within the body. Since MN-PCE induction peaks between 24 and 48 h post-treatment in most cases, the slow elimination of MDI metabolites may be important in the observation of increased MN-PCE at 7 days after the last MDI exposure.

The formation of MN is a consequence of chromosomal breakage and/or spindle-fiber dysfunction induced by clastogens and/or aneuploidogens. They are small, contain chromatin, exist within the cytoplasm, and are separated from the main nucleus following mitosis. MN formation in bone marrow polychromatic erythrocytes has often been used as a genetic endpoint. Results of the present study showed that MN were induced in bone marrow polychromatic erythrocytes in a dose-related manner after exposure of rats to 7.1 and 113.2 mg/m^3 MDI. The ratio of NCE/PCE was not different between control and exposure groups, suggesting that there was no observable bone marrow cytotoxicity. At least 2 potential mechanisms

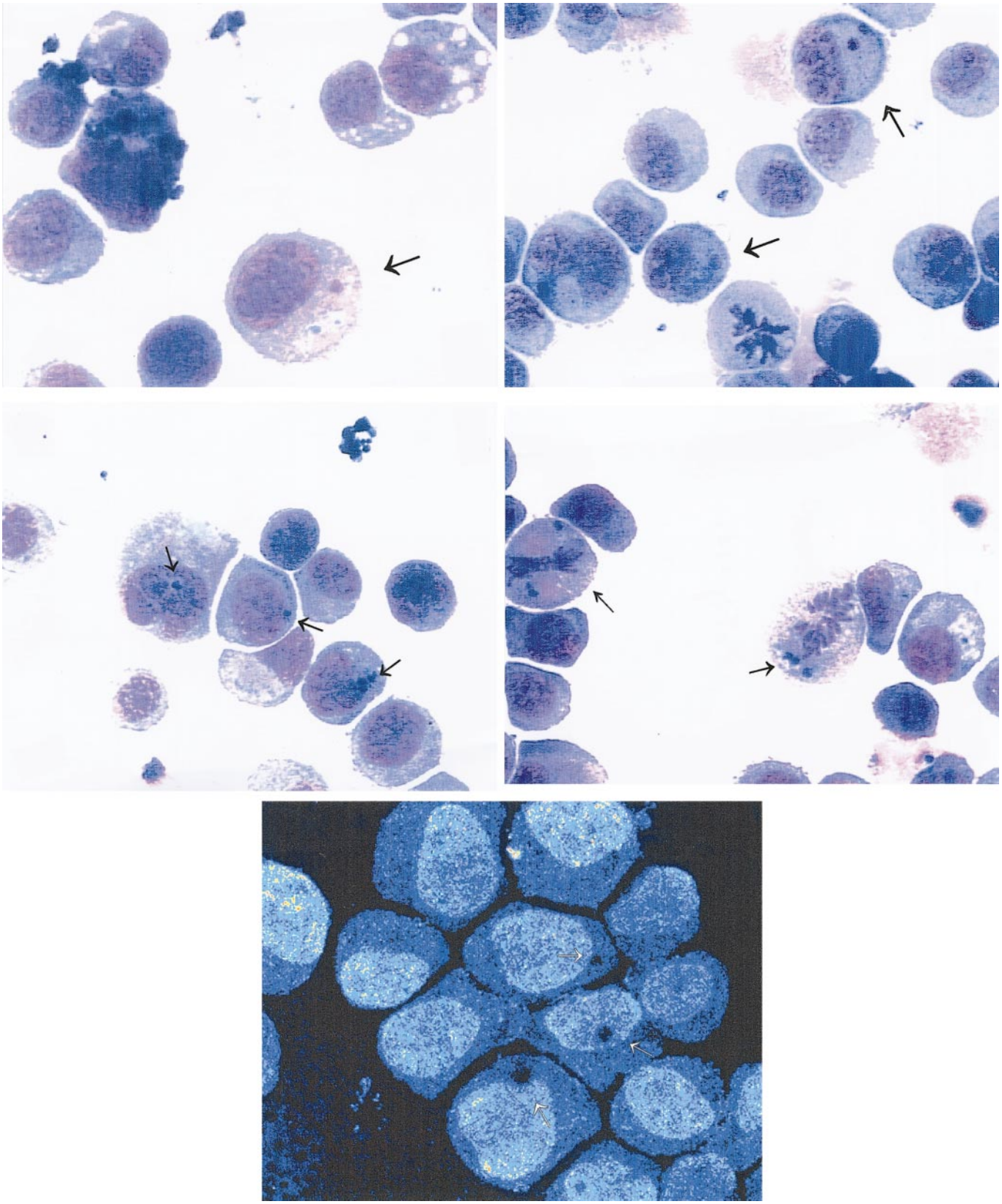

FIG. 2. Photomicrographs of fibroblast cells exposed to MDI-GSH displaying MN and MN-GSH particulate inside the cells at various stages of cell division. Magnification ×700. Upper left panel, fibroblast with 1 MN (arrow); upper right panel, 2 MN within an individual fibroblast (arrows); center left panel, fibroblasts with particulate within the nuclei and cytoplasm (arrows); center right panel, fibroblasts in metaphase with particulate in cytoplasm (arrows); bottom panel, MDI-GSH particulate present in confocal microscopy (the arrows point to particles within the cytoplasm and nucleus).

TABLE 3
Effect of MDI-GSH Conjugate on V 79 Cell Division

Treatment	Concentration (μg/ml)	Cells in metaphase[a] (mean ± SD)	Cells with intracellular precipitant granules[a]	Nuclear division index
DMSO control	0.0	11 ± 5	0	1.01
MDI-GSH	100	19 ± 11*	334 ± 55**	1.02
MDI-GSH	250	27 ± 13**	416 ± 42**	1.02
MDI-GSH	500	42 ± 14**	520 ± 39**	1.03
MDI-GSH	1000	34 ± 16**	270 ± 76**	1.02

[a]Values = number/1000 cells. The data was averaged from 2 experiments.
*$p < 0.05$, ** $p < 0.01$ compared to DMSO control.

exist that may be responsible for the MN results observed in the studies. Hydrolysis 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 sulfur to nitrogen, or sulfur to oxygen exchange and 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* and 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 μg/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 μg/ml. MDA was detected in the supernatant and sediments with 20% and 80% in each fraction, respectively. Dose-dependent induction of MN following incubation of the cells with MDI-glutathione was observed; however, the slope of the curve was rather shallow. Dose dependency of MN induction to the MDI-cysteine conjugate was not as clearly established, possibly due to the greater toxicity of this conjugate compared to the MDI-GSH conjugate. MDA was not detected from these cultures, indicating that the mechanism of MN induction was not via hydrolysis of MDI to MDA. It is unknown whether the MDI-thiol conjugate effects are via DNA, or via protein-adduct formation and possibly reaction with spindle components. The observed intracellular and intranuclear precipitants and increased percentage 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 for immunological studies in our laboratory because of its tendency to produce high levels of IgE. There is, in the rat bone marrow MN test, severe interference due to the great numbers of basophilic mast granules that usually are difficult to distinguish from MN, especially in the Fisher-344 strain (MacGregor *et al*, 1987). The Brown Norway rat bone marrow contains very few of these free mast granules and thus can be assessed without purification (Fig. 1). The lack of interference from mast cell granules was confirmed using acridine-orange staining of bone marrow smears from control and Brown Norway rats exposed to MDI or vincristine. The relative sensitivity to genotoxic agents of this strain vs. others that have been used for MN studies is not known.

In summary, the present research has demonstrated that inhalation of MDI aerosol produces dose-dependent genetic toxicity in the Brown Norway rat. MDA, MDI-cysteine, and MDI-glutathione 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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