Environmental Health Perspectives
Vol. 72, pp. 177-182, 1987

Evaluation of Sister Chromatid Exchange and Cytotoxicity in Murine Tissues *In Vivo* and Lymphocytes *In Vitro* Following Methyl Isocyanate Exposure

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The purpose of this study was to assess sister chromatid exchange (SCE) levels and cell cycle kinetics in various murine tissues following MIC exposure. Following exposure of mice to MIC, these parameters were measured in bone marrow and alveolar macrophages labeled with BrdUrd *in vivo* and in peripheral blood and spleen lymphocytes cultured in the presence of BrdUrd *in vitro*. Target concentrations of MIC were 2, 15, and 30 ppm (3 hr).

Neither elevated SCE frequencies nor inhibition of cell cycling were evident in lipopolysaccharide (LPS)- or concanavalin A (ConA)-stimulated spleen lymphocytes, or in LPS-stimulated peripheral blood lymphocyte (PBL) cultures from mice exposed for 3 hr to MIC concentrations as high as 30.5 ppm. Inhibition of cell cycling and poor culture success rates were apparent in ConA-stimulated PBLs following MIC exposures as low as 2.3 ± 0.4 ppm for 3 hr.

At the lowest MIC dose employed, the cycling characteristics of bone marrow and alveolar macrophages were not altered, and SCE frequencies were at control levels. However, severe cell cycle inhibition was observed in these tissues at MIC concentrations of 15 ppm or greater. A marker of cytotoxicity at this dose was a high frequency (approximately 33–90%) of occurrence of first division cells containing a late-replicating Y chromosome.

Despite its apparent cellular toxicity, MIC is not genotoxic as measured by SCE analysis in the tissues examined in this study.

Introduction

The Bhopal tragedy focused attention on the lack of methyl isocyanate (MIC) toxicity data and stimulated interest in the evaluation of acute and long-term toxicological effects of MIC. Of particular interest to Bhopal survivors are the potential genetic hazards associated with MIC exposure. Cytogenetic studies, including sister chromatid exchange (SCE) analysis, have been carried out in cultured peripheral blood lymphocytes (PBLs) of MIC-exposed patients, but limited data were reported (*1*).

SCE is a sensitive indicator of chromosomal damage produced by electrophilic alkylating agents (*2*). Because MIC is an electrophilic acylating agent, it might be expected to induce SCEs in mammalian tissues. The SCE methodology also permits study of cell cycle kinetics. The present study was undertaken to assess SCE levels and cytotoxicity in various tissues of male BDF_1 mice following inhalation of MIC. Tissues examined in this study include cultured spleen and PBLs, as well as alveolar macrophage and bone marrow cells.

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Materials and Methods

Animals

Male, BDF_1 mice (3–3.5 months old, 26–32 g) were used in this study. The parental breeders (C57B1/6J and DBA/2J) were purchased from Jackson Laboratory (Bar Harbor, Maine).

Chemical

Methyl isocyanate was purchased from Aldrich Chemical Company (Milwaukee, WI) and used as received (Lot # 3317 AL was used).

MIC Exposures

MIC exposures were carried out as described by Ferguson et al. (*3*). The desired exposure concentrations (2.3–30.5 ppm, 3 hr) were obtained by evaporation of MIC (10 mL) held in a 500 mL bottle maintained at 0°C in an ice bath. The evaporating bottle was sealed with a rubber septum covered with a Teflon film (Supelco, Septa No. 3-3200, Bellefonte, PA). A 16-gauge hypodermic needle (38 mm in length) was passed through the septum from the inside and then attached to a polyethylene tube (PE-190), 160 mm in length, for delivery of MIC vapor to the exposure chamber. An 18-gauge hypodermic needle (50 mm in length) penetrated the system from the outside for constant delivery of dry air to the evaporating bottle. This needle was connected to a Gilmont microflowmeter (No. J-4184) equipped with a micrometer valve to regulate air delivery between 0.02 and 15 mL/min.

To generate MIC concentrations between 2.3 and 30.5 ppm, the air delivery to the evaporating bottle was set at 1.0 mL/min, and the airflow in a central mixing chamber varied between 7 and 42 L/min. Four glass exposure chambers were connected to the mixing chamber, with 2 L/min of air being pulled through each chamber from the central chamber. Before mice were placed in the system, analysis of MIC concentration in the exposure chambers were conducted to assure that the concentrations in the chambers were consistent and stable. Six to ten mice were placed in one or more chambers to start the exposure. During the 3-hr exposure, samples were taken every 3 to 5 min for MIC analysis.

Analytical Determination of MIC Exposure Concentration

A Perkin-Elmer Model 3920 gas chromatograph equipped with a nitrogen-phosphorus detector was used. A glass column (1/4 in O.D. × 6 ft length) permitted on- column injection of samples. The column was packed with 3% OV- 210 on Chromosorb WHP (80/100 mesh) and used isothermally at 60°C. Helium passed through the column at 20 mL/min (inlet pressure: 60 psi). The injector and interface temperatures were both set at 200°C. Air and hydrogen passed into the nitrogen-phosphorus detector at 30 mL /min and 1 to 3 mL/min, respectively. MIC gas standards were prepared by injecting known volumes of pure MIC gas into glass vessels of 1000 mL in volume, thus generating dilutions. These vessels contained glass beads for mixing and were equipped with Teflon-coated septa, enabling the withdrawal of desired volumes for subsequent injection into the chromatograph. Gas-tight (Unimetrics) syringes were used for injections of volumes between 100 and 1000 μL. The absolute retention time for MIC under these conditions was approximately 1.0 min, and 0.8 ng was detectable. Following calibration, samples were drawn from the animal exposure chamber with the same syringes. Samples were taken approximately every 3 to 5 min during exposure, and the mean concentration was determined for each exposure. Coefficients of variation for each mean were under 20%, indicating low variation between samples analyzed during an exposure.

Lymphocyte Sampling and Culture

Immediately following each exposure, blood was drawn via orbital sinus bleeding of four to six exposed and two to four nonexposed control mice. Peripheral blood lymphocyte (PBL) sampling and culture were as previously described (*4,5*). Approximately 0.65 to 0.95 mL of blood was collected from each mouse by orbital sinus bleeding. PBLs were isolated on Histopaque 1077 (Ficoll) (Sigma), washed three times with sterile phosphate-buffered saline containing 2% heat-inactivated fetal bovine serum (wash solution), stained with Turk's solution, and counted with a hemacytometer. The cells were grown in 24-well tissue culture plates (Linbro: Flow Laboratories). Each culture was inoculated with 7×10^5 to 10^6 cells. The cells were grown in RPMI 1640 (Gibco), 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine (R.C. Biologicals), and penicillin-streptomycin (Gibco). Also, 5-bromo- 2'-deoxyuridine (BrdUrd, Sigma) at a final concentration of 3 μM, and a mitogen, lipopolysaccharide (LPS) (60 μg/mL; Sigma) or concanavalin (ConA) (6 μg/mL; Sigma Type IV) were added to the cultures in order to stimulate mitogenesis of B or T lymphocytes, respectively. In addition, all cultures contained 2.5×10^{-5} M 2-mercaptoethanol to enhance mitogenesis. The complete media volume was 1 mL. The cultures were maintained in darkness at 37°C and 5% CO_2. At 20 to 24 hr after initiation of cultures, an additional 1 mL of complete culture media was added to each well. Cells were harvested as described by Erexson et al. (*6*) after a total of 48 hr incubation. Colcemid (0.05 mL of a 10 μg/mL solution) was added 3 hr prior to harvest.

After orbital sinus bleeding, mice were sacrificed by cervical dislocation, and the spleens were removed and transferred to sterile conical centrifuge tubes containing 3 mL of wash solution. Spleen cells were suspended by gently smashing the spleen with a sterile spatula. Spleen suspensions were not separated on Histopaque, but all subsequent washings, cell counts, and culture were identical to that described for PBLs.

BrdUrd Labeling of Bone Marrow and Alveolar Macrophages *In Vivo*

Immediately following MIC exposure, mice (4–6 exposed and 2–4 controls) were infused IV with BrdUrd (10 mg/mL, flow rate 3.6 mL/24 hr) by an infusion device described by Conner et al. (*7*). Infusion of BrdUrd was for 17 hr plus 4 hr colchicine (bone marrow and alveolar macrophages) or for 19 hr plus 2 hr colchicine (bone marrow only). Harvest of bone marrow and alveolar

macrophage cells was as previously described by Conner et al. (*8*).

Evaluation of Slides

All slides were stained by a modification of the fluorescence plus Giemsa (FPG) method (*9*). Slides were coded and scored blindly. SCE was scored in 20, second-division metaphases per each culture or cell type per animal. In addition, 100 consecutive metaphases were analyzed for cell cycle kinetic analysis.

Results

Following exposure of mice for 3 hr to MIC concentrations of 2.3 ± 0.4, 15.3 ± 1.6, or 30.5 ± 4.3 ppm, SCEs and cell cycle kinetics were evaluated in ConA- and LPS-stimulated spleen lymphocytes and PBLs (Table 1). In all cases, variances among treated and control groups were determined to be significantly heterogeneous. In order to minimize the effect of unequal variances, a square root transformation of SCE data was performed (*10*) prior to one-way analysis of variance (ANOVA). No significant differences ($p = 0.5$) were apparent among the SCE responses in LPS-stimulated spleen lymphocytes of control mice and of mice exposed for 3 hr to MIC concentrations of 2.3 ± 0.4 to 30.5 ± 4.3 ppm. Likewise, no significant differences in SCE responses were observed among the ConA-stimulated spleen lymphocytes of control and exposed mice. Similarly, in LPS-stimulated PBLs, as well as in ConA-stimulated PBLs, no significant differences were detected among SCE responses of MIC-exposed mice and their respective controls.

Table 1. SCE levels and cell cycle kinetics in cultured lymphocytes of mice exposed to MIC.

Cell type	Dose, ppm, 3 hr	Number of mice[a]	Mean SCE ± SD	Relative percentage of first/second/third division cells
PBL-ConA	0	11(12)	8.9 ± 0.92	39.6/31.3/29.1
	2.3 ± 0.4	2(5)	10.2 ± 0.28	63.5/23/13.5
	15.3 ± 1.6	2(12)	9.1 ± 1.8	26/22/51
	30.5 ± 4.3	2(10)	10.8 ± 0.64	45.5/42/12.5
PBL-LPS	0	9(9)	7.4 ± 1.5	36.8/40.4/22.8
	2.3 ± 0.4	5(5)	6.3 ± 0.92	41.2/38.4/20.4
	15.3 ± 1.6	8(8)	7.2 ± 1.8	41.1/34.8/24.1
	30.5 ± 4.3	10(10)	6.8 ± 0.60	36.3/39.8/23.9
Spleen-ConA	0	9(9)	8.7 ± 1.6	12.1/17.2/70.7
	2.3 ± 0.4	5(5)	9.4 ± 0.61	13/18.6/68.4
	15.3 ± 1.6	9(9)	11.0 ± 1.3	9.8/12.3/77.9
	30.5 ± 4.3	5(5)	9.1 ± 0.54	14.2/22/63.8
Spleen-LPS	0	9(9)	8.3 ± 1.6	19.4/30/50.6
	2.3 ± 0.4	5(5)	9.2 ± 0.98	17.4/31.6/51
	15.3 ± 1.6	9(9)	10.2 ± 1.5	19.9/32.8/47.3
	30.5 ± 4.3	5(5)	10.2 ± 2.2	12.6/32.2/55.2

[a] Twenty cells/mouse were scored for SCE and 100 cells/mouse for cell cycle kinetics. Numbers in parentheses are the number of cultures initiated.

The only notable treatment effect observed in cultured lymphocytes of MIC-exposed mice was severe toxicity exhibited in ConA-stimulated PBL cultures over the entire range of MIC concentrations employed in this study. The success rates for ConA-stimulated cultures were 2/5 at 2.3 ± 0.4 ppm, 2/12 at 15.3 ± 1.6 ppm, and 2/10 at 30.5 ppm. A successful culture is one that provides a minimum of 20 good quality second-division metaphases for SCE counting and at least 100 total metaphases for cell cycle kinetic analysis. In the unsuccessful cultures, blastogenesis was apparent, but the c-metaphase yield was drastically reduced. The decreased mitotic yields in the ConA-stimulated blood lymphocyte cultures were not the consequence of low cell densities in the cultures, as all wells were seeded with 7.5×10^5 to 10^6 Ficoll-separated lymphocytes. Although the total yield of Ficoll-separated lymphocytes was generally lower (ANOVA, $p < 0.005$) in the MIC-exposed mice relative to control mice (Table 2), all LPS-stimulated cultures were successful. No culture failures were observed in ConA-stimulated spleens, and only 1 of 12 were unsuccessful in ConA-stimulated PBL controls.

As illustrated in Table 1, inhibition of cell cycling was observed in ConA-stimulated blood lymphocytes from mice exposed to an MIC concentration as low as 2.3 ± 0.4 ppm. An increase in the percentage of first-division metaphases, as well as decreases in the percentages of second and third or higher division metaphases, were observed in the low dose exposure group. By contrast, the cell cycle kinetics of LPS-stimulated PBLs, as well as of LPS- or ConA-stimulated spleen lymphocytes of mice exposed to MIC concentrations as high as 30.5 ppm for 3 hr, were not significantly (chi-square, $p = 0.05$) inhibited from the cell cycle kinetics of their respective controls.

SCE and cell cycle kinetics analyses were also carried out for bone marrow cells and for alveolar macrophages lavaged from the lungs from control and MIC-exposed (2.3 ± 0.4 ppm, 3 hr) mice following *in vivo* labeling with BrdUrd immediately after MIC exposure. At the low exposure dose, neither the mean SCE levels (ANOVA, $p < 0.01$) nor cell cycle kinetics (chi-square, $p = 0.05$) of bone marrow or alveolar macrophages differed significantly from their respective control SCE baseline and cell cycle kinetics.

Cell cycle kinetics were also assessed in bone marrow and alveolar macrophage cells in a preliminary study of mice exposed for 3 hr to 15.3 ± 1.6 ppm MIC (Table

Table 2. Ficoll-separated peripheral blood lymphocyte counts/mL blood.

MIC dose, ppm, 3 hr	Mean lymphocyte count ± SD × 10^6 mL blood
0	3.9 ± 1.1
2.3 ± 0.4	2.3 ± 0.68
15.7 ± 1.6	2.24 ± 0.65
30.5 ± 4.3	2.30 ± 0.57

3). Severe cell cycle inhibition occurred at this dose. All bone marrow and alveolar macrophage metaphases from exposed mice were arrested at their first cell cycle. Examination of 50 cells of each type per exposed mouse revealed the presence of a single lightly stained chromosome; the remaining chromosomes were darkly stained in 89.6% of bone marrow and 80% of alveolar macrophage cells (Fig. 1). The origin of the lightly stained chromosome may be explained as follows: Early replicating chromosomes incorporate thymidine during DNA synthesis (prior to BrdUrd infusion), and consequently, stain darkly at the first metaphase. By contrast, a late replicating chromosome in the same cell commences DNA synthesis following the start of BrdUrd infusion and subsequently stains lightly at the first metaphase. A reduced mitotic index, determined in bone marrow cells, was consistent with cytotoxicity at this dose. Mitotic indices were not evaluated for alveolar macrophages, as the indices are generally very low in this tissue.

In order to further study the MIC-dose dependence of the lightly stained chromosome phenomenon, mice were exposed to a slightly lower MIC concentration (12.2 ± 1.6 ppm, 3 hr). In addition, a shorter colchicine treatment (2 hr) was used to produce less condensed chromosomes. Because of this short colchicine treatment, only bone marrow slides were prepared for analysis. At 12.2 ± 1.6 ppm, the mitotic index of exposed mice was not reduced relative to control mice. However, a significant cell cycle delay (chi-square, $p = 0.05$) occurred in MIC-exposed mice. Exposed mice also exhibited a distribution of first division cell types (Table 3), which differed significantly from that of control mice (chi-square, $p = 0.05$). Exposed mice had fewer normal first division cells than did control mice. Relative to mice exposed to 15.3 ± 1.6 ppm in the preliminary studies, mice exposed at 12.2 ± 1.6 ppm had fewer first division cells containing the lightly stained chromosome. Controls had a negligible number of first-division cells containing the lightly stained chromosome.

Only bone marrow cells were examined in another group of mice exposed to 15.7 ± 1.0 ppm MIC. Extreme variation in cell cycle kinetics was noted in this exposure group (1 mouse: 26/69/5; 1 mouse 100/0/0). Mean cell cycle parameter data are presented in Table 3. Although the mean mitotic index of the exposed bone marrows is not reduced relative to the control group, cell cycle kinetics and the first division cell-type distribution differ significantly (chi-square, $p = 0.05$) from respective control values. A considerable percentage (33.6%) of first division cells contain the single lightly stained chromosome. Severe cell cycle inhibition is associated with the occurrence of the light Y chromosome in a first division cell. The occurrence of such cells implies that their subsequent transit time in late S and G_2 phases has been extended to 17 to 21 hr (time of BrdUrd infusion plus colchicine). Examination of Table 3 reveals that the appearance of this chromosome appears to be MIC dose-related. Furthermore, the data suggest that the dose-effect curve for the light Y chromosome is very steep, and the threshold for its occurrence is >15 ppm.

Discussion

The primary purpose of this study was to assess SCE levels and cytotoxicity in various murine tissues following MIC exposure. Tissues chosen for analysis were either exposed directly to MIC (alveolar macrophages) or were exposed indirectly via absorption into the blood (PBLs) and transport to remote tissues (spleen lymphocytes and bone marrow cells). Assessment of SCEs in spleen and PBLs requires incorporation of BrdUrd during *in vitro* culture, whereas BrdUrd labeling of bone marrow and alveolar macrophages is accomplished *in vivo*. In any case, exposure of mice for 3 hr to MIC over the concentration range of 2.3 ± 0.4 to 30.5 ± 4.3

Table 3. Cell cycle kinetics and SCE frequencies in bone marrow and alveolar macrophage cells of mice exposed to MIC.

MIC dose, ppm, 3 hr	Number of mice	Cell type[a]	Mitotic index[b]	Percentage of $M_I/M_{II}/M_{III}$ cells[c]	M_{II} SCE	Percentage of M_I cells with light Y chromosome
0	6	BM	119 ± 12.7	25.8/60.5/13.7	3.4 ± 0.34	—
	6	AM	—	50.5/46.8/2.7	3.6 ± 0.64	—
15.3 ± 1.6	5	BM	22 ± 7.1	100/0/0	—	89.6
	5	AM	—	100/0/0	—	80
0	4	BM	42.5 ± 12.9	29.5/56.8/13.8	3.8 ± 1.1	0.7
12.2 ± 1.6	4	BM	44 ± 16	37.5/58/4.5	3.9 ± 0.7	6
15.7 ± 1.0	4	BM	44.8 ± 17.8	74/24.8/1.2	3.1 ± 2.1[d]	33.6

[a] BM-bone marrow, AM-alveolar macrophages. In the experiments where BM and AM data are given, cells were harvested after 17 hr of BrdUrd infusion plus 4 hr colchicine. In experiments where only BM data are given, cells were harvested after 19 hr of BrdUrd infusion plus 2 hr colchicine.

[b] Number of c-metaphases ± SD per 1000 cells.

[c] M_I - first division, M_{II} - second division, M_{III} - third division cells.

[d] With the exception of one mouse, M_{II} = 15%, and the total number of M_{II} cells was not adequate for analysis. The mean SCE frequency is calculated for the one mouse for which an adequate yield of M_{II} cells was obtained.

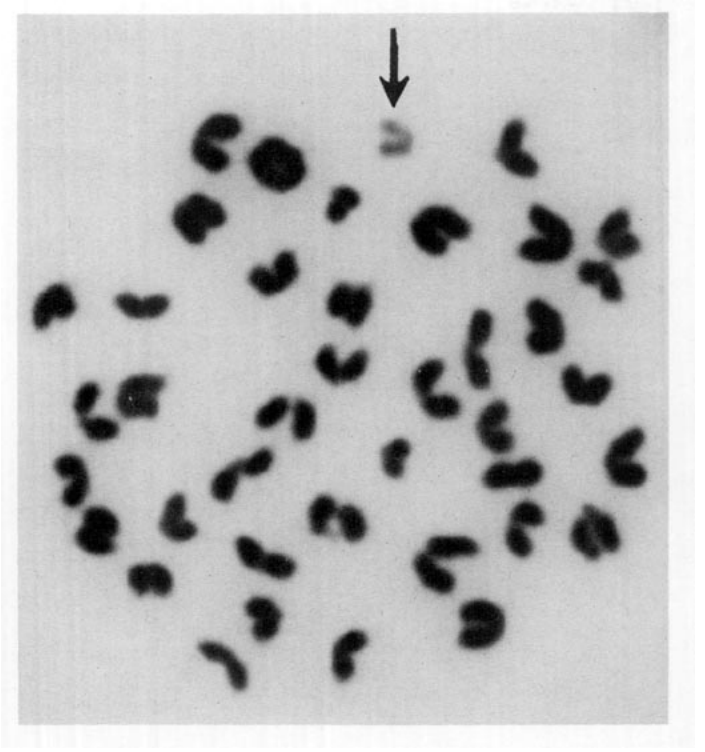

FIGURE 1. First division cells containing the late-replicating lightly stained Y chromosome, denoted by an arrow.

ppm was not effective in producing SCE frequencies in cexcess of baseline SCE levels in cultured lymphocytes. Cytotoxicity of MIC precluded assessment of SCE in bone marrow and alveolar macrophages at the intermediate and high doses.

In addition to SCE analysis, BrdUrd incorporation provides a means for assessment of inhibition of cell cycling (*11*). No evidence of cell cycle inhibition was found in LPS- or ConA-stimulated spleen lymphocytes or in LPS-stimulated PBL cultures from mice exposed to MIC concentrations as high as 30.5 ppm (3 hr). In contrast, inhibition of cell cycling of ConA-stimulated PBL was apparent even at the lowest MIC concentration (2.3 ± 0.4 ppm, 3 hr). Poor growth of ConA PBL cultures was consistently observed at all MIC doses but not in control cultures. These results suggest that either MIC or a by-product of MIC enters the peripheral circulation and selectively affects T lymphocytes in general or affects a subpopulation of T lymphocytes. The toxic effect of MIC on T lymphocytes from blood appears to occur after the blast transformation stage, as blast cells were abundant on slides prepared from these cultures. Further studies are required to elucidate the mechanism of the specific peripheral blood T-lymphocyte toxicity induced by MIC.

MIC-induced cytotoxicity is not limited to cultured peripheral T lymphocytes. Dramatic cell cycle inhibition was also observed in remote bone marrow and alveolar macrophage cells of mice exposed to MIC concentrations in excess of 15 ppm (3 hr). A marker of severe cell cycle inhibition in bone marrow and alveolar macrophages was the occurrence of primarily first division metaphases, the majority of which contained a single, lightly staining chromosome and the other, darkly staining chromosomes. This staining pattern is expected in cells in which all except one chromosome complete synthesis of the first cycle prior to BrdUrd treatment, and the single remaining chromosome replicates once in the presence of BrdUrd. The Y chromosome in male murine somatic cells is described as late-replicating (*12*) and has been distinguished by BrdUrd-labeling methods (*13*). The lightly stained chromosome observed in the present study is most likely the late-replicating murine Y chromosome. At MIC concentrations in excess of 15 ppm, very severe cell cycle inhibition is evident from the fact that 17 to 21 hr (BrdUrd infusion time plus colchicine) are required for completion of replication of the Y chromosome and subsequent progression of the cell cycle to its first metaphase.

The mechanism of the cytotoxicity produced by MIC, as well as any potentially detrimental consequences of the light Y chromosome, remains to be established. Although the present study does not indicate significant MIC genotoxic activity as measured by SCE analysis, our findings illustrate the utility of BrdUrd labeling for assessment of general cellular cytotoxicity.

This study was supported under Grants 1-R01-CA 33869-01 and 1-R01-ES02747 from the National Institute of Environmental Health Sciences. We thank Suzanne Bertera for photographic assistance.

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