Environmental Health Perspectives
Vol. 72, pp. 143-148, 1987

Myelotoxicity Induced in Female B6C3F1 Mice by Inhalation of Methyl Isocyanate

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The effects of a 4-day inhalation exposure (6 hr/day) to 0, 1, and 3 ppm methyl isocyanate (MIC) on bone marrow parameters in female mice were examined at 5, 8, and 21 days following exposure. The MIC exposure was associated with myelotoxicity as evidenced by hypocellularity, suppression of pluripotent stem cells (CFU-S), granulocyte-macrophage progenitors (CFU-GM) and erythroid precursors (CFU-E) in both dose groups. Hematopoietic parameters returned to normal by 21 days in the 1 ppm dose group, but not in the 3 ppm dose group. This indicates that the alterations in the bone marrow parameters persist for a relatively long period at dose levels where there are little or no changes in body weight, clinical pathology, or immunological parameters, suggesting that the bone marrow may be a sensitive endpoint for MIC exposure in mice. MIC is a highly reactive chemical that appears to exert its effect directly on the lining epithelium of the nasal cavity and major airways; there was no histological evidence of a systemic effect. The pathogenesis of the bone marrow depression is unknown; however, there were chronic bronchitis and bronchial fibrosis in the 3 ppm dose group. One possible explanation is that the cell injury induced in the lung is associated with the release of inhibitory factors for hematopoiesis, as the rodent lung is a potent source of both stimulatory and inhibitory growth factors for bone marrow progenitor cells. A second possibility is that the thymic atrophy found in MIC-exposed mice might be related to myelotoxicity. The pathogenesis of myelotoxicity in MIC exposure and its relationship with pulmonary injury require further study.

Introduction

Methyl isocyanate (MIC) is an extremely reactive, toxic, volatile, and flammable compound which exists as a liquid at room temperature (*1*). It is used as an intermediate in the manufacture of a variety of carbamate pesticides (*2*). Kimmerle and Eben (*3*) summarized some of the acute toxic properties of MIC, and noted the irritating nature of MIC to skin and mucous membranes. Irritation such as coughing and nasal secretion has been reported in workers exposed to isocyanates (*4,5*). The studies of Pozzani and Kinkead (*6*) indicated similar symptoms in rats and mice exposed to MIC.

The accidental release of MIC in Bhopal, India, December, 1984 (*7*), resulted in deaths and exposure of a large number of people to MIC and led the World Health Organization to request that the National Toxicology Program investigate more extensively the potential toxic effects of the chemical. Since the bone marrow is often a sensitive indicator of toxicity, it was decided to evaluate hematopoietic progenitor cells following exposure to MIC. Female B6C3F1 mice were chosen as this is the sex and species that we have used in our previous studies on the effect of environmental chemicals in myelotoxicity. The response of the bone marrow to sublethal doses was evaluated because it was felt that myelotoxicity at dose levels that caused mortality would be considered meaningless. The present study reports the effects of MIC exposure on bone marrow cellularity and hematopoietic progenitor cells. This includes pluripotent bone marrow stem cells as measured by colony forming units in the spleen of irradiated recipients (CFU-S), *in vitro* granulocyte-macrophage progenitors (CFU-GM), and erythroid progenitors (CFU-E). These results are correlated with the pulmonary damage found histologically during the postexposure recovery phase.

Materials and Methods

Mice

Female B6C3F1 (C57BL/6N × C3H) mice, 10 weeks of age and weighing 18 to 22 g, were obtained through Charles River (Kingston, NY) and were quarantined for 10 to 21 days prior to random distribution to exposure groups. Exposures to 0, 1, or 3 ppm MIC were carried out for 6 hr/day on 4 consecutive days in stainless-steel exposure chambers. The details of vapor generation, monitoring, and safety aspects have been reported by Adkins et al. (*8*). Exposures were conducted from 6-17 to 6-20-85. Following exposure, the mice were

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Table 1. Body and organ weight ratios 7 days after exposure to MIC.[a]

Exposure group, ppm	Weights						
	Body (g)	Liver[b]	Spleen[b]	Kidney[b]	Thymus[b]	Lung[b]	Brain[b]
0	19.5 ± 0.6	55 ± 2	3.6 ± 0.2	7.0 ± 0.2	2.9 ± 0.1	7.8 ± 0.3	24 ± 1
1	19.9 ± 0.6	52 ± 1	3.2 ± 0.1*	6.1 ± 0.1	2.6 ± 0.1	7.5 ± 0.2	23 ± 1
3	19.0 ± 0.4	52 ± 1	2.9 ± 0.1**	6.3 ± 0.1*	2.4 ± 0.1**	8.2 ± 0.6	23 ± 1

[a] Mean ± SEM, $N = 5$ female B6C3F1 mice.
[b] Organ/body weight ratio × 10^3.
* $p < 0.05$ vs controls.
** $p < 0.01$ vs controls.

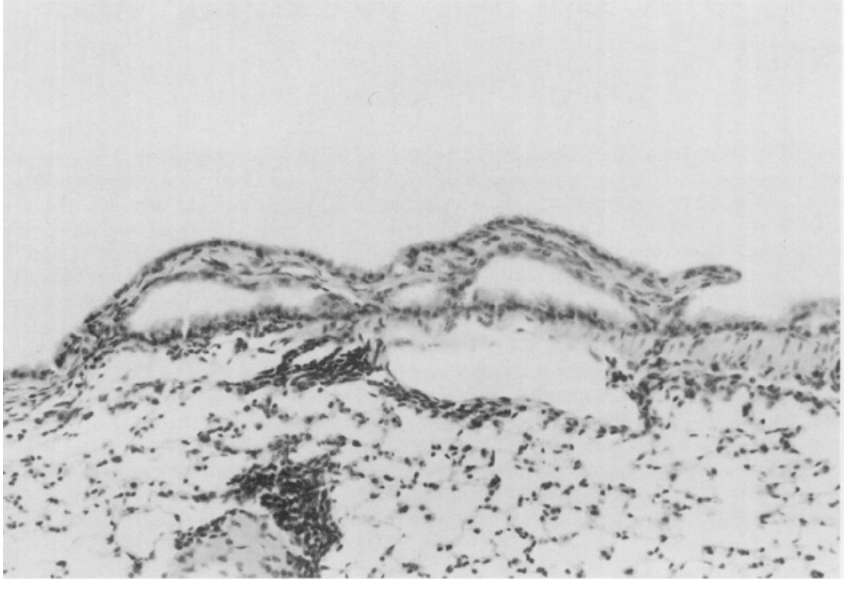

FIGURE 1. Intraluminal fibrosis in the major bronchus of a female B6C3F1 mouse exposed to 3 ppm MIC.

housed five per cage and allowed free access to commercial rodent chow (NIH-07 rodent diet, Ziegler Bros. Inc., Gardner, PA). The hematopoietic parameters were measured on days 5, 8, and 21 following exposure. Histopathological examinations were performed on days 7, 28, 49, and 91 of postexposure.

Histopathology

Mice were given an overdose of Nembutal IP and subjected to a complete pathological examination. The body weight, thymus, liver, brain, right kidney, and lung weights were recorded. Tissues were fixed in buffered formalin, and selected tissues (nasal passages, liver, gall bladder, two levels of trachea, four lobes of lung, thyroid, brain kidneys, eyes, thymus, spleen, heart, and stomach) were examined histologically.

Bone Marrow Cellularity

Marrow cells were aseptically collected by dissecting both femurs free of attached tissue, cutting the femur at the epiphysis, and flushing the shaft with 1.5 mL of RPMI-1640 (Flow Laboratories, McLean, VA). Single cell suspensions were prepared by successive passages of the cells through 22- and 25-gauge needles. Nucleated cells were enumerated by the Coulter Model ZB Counter (Coulter Electronics, Inc., Hialeah, FL).

Granulocyte-Macrophage Progenitors (CFU-GM). These progenitors from the bone marrow were assayed by using established semisolid culture techniques (*9*). Briefly, 10^5 nucleated femoral marrow cells in 1 mL of RPMI-1640 culture medium were plated in 35 × 10 mm Falcon tissue culture dishes (Falcon Plastics, Oxnard, CA). The medium was supplemented with 1.5% methylcellulose, 20% fetal bovine serum, 5% human AB serum (Flow Laboratories, McLean, VA), 2 mM L-glutamine, 0.1% gentamicin, and 10% mouse lung conditioned medium. Culture plates were incubated at 37°C in a humidified atmosphere containing 7% CO_2 for 7 days. The plates were stained with methylene blue, and total colonies (40 or more cells) per plate were counted using a stereomicroscope (Wild, Heerbrugg, Switzerland).

Colony-Forming Units in Spleen (CFU-S). The assay for multipotential stem cells using the spleen colony method was performed on mice 5 days following the final exposure, as described previously (*9*). The recipi-

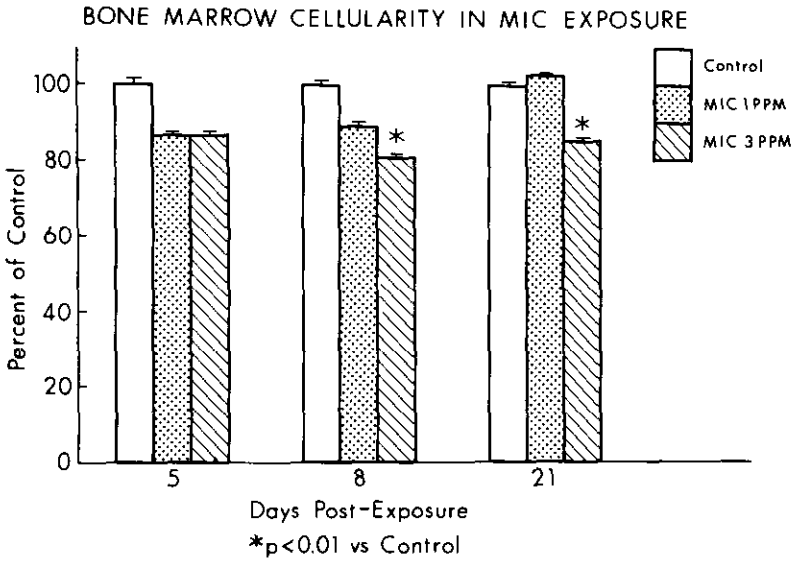

FIGURE 2. Effect of MIC on bone marrow cellularity. These assays were determined at 5, 8, and 21 days following a 4-day inhalation of 0, 1, and 3 ppm MIC to 10 female B6C3F1 mice per group. The bone marrow cellularity was expressed as marrow nucleated cells/femur and was given as the percent of control. (*) denotes significant difference from controls ($p < 0.01$).

Table 2. Pluripotent stem cells and erythroid precursors following MIC exposure.[a]

Exposure group, ppm	CFU-S[b]		CFU-E[c]	
	CFU-S/ 5×10^4 cells	CFU-S/femur ($\times 10^2$)	CFU-E/ 2×10^5 cells	CFU-E/femur ($\times 10^2$)
0	17.5 ± 0.2	90 ± 1	87.6 ± 0.6	94 ± 1
1	15.6 ± 0.2*	69 ± 1* (23% ↓)	81.1 ± 0.2*	78 ± 1* (17% ↓)
3	14.1 ± 0.3*	63 ± 1* (30% ↓)	73.1 ± 0.4*	65 ± 1* (31% ↓)

[a] Mean ± SEM, $N = 5$ female B6C3F1 mice.
[b] The assay of pluripotent stem cells (CFU-S) was performed on day 5 postexposure.
[c] The assay of erythroid precursors (CFU-E) was performed on day 8 postexposure.
* $p < 0.01$ vs. controls.

ent B6C3F1 female mice had received 600 rads of total body irradiation at 273 rads/min (^{137}Cesium Irradiator, Model 431, J. L. Shepherd and Assoc., Glendale, CA). Each recipient received 5×10^4 nucleated bone marrow cells intravenously within 24 hr of irradiation. Four recipients were used for each donor. Eight days later, the spleens were removed, placed in Bouin's fixative, and the spleen colonies were enumerated.

Colony-Forming Units Erythroid (CFU-E). The erythroid precursors in the bone marrow from the mouse were determined by using a modification of Iscove's method (*10*). Nucleated femoral marrow cells (2×10^5) in 1 mL of culture medium, consisting of 1 × alpha modification of Eagle's medium with Earle's salts, 30% fetal bovine serum (Flow Laboratories), 1.5% methylcellulose, 10^{-4} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 0.25 units/mL erythropoietin (Connaught Labs., Swiftwater, PA), were incubated in a CO_2 incubator (described above) for 6 days. Colonies were stained with a modified benzidine method (*11*) and counted under the stereomicroscope. Five plates were counted for each bone marrow determination.

Statistical Analysis

Data were analyzed by the RS/1 multicompare procedure, using the Wilk-Shapiro test for normality, one-way analysis of variance, and Dunnett's test for multiple comparison with a control group (*12*).

Results

Pathology

The acute MIC exposure by inhalation at 0, 1, or 3 ppm for 6 hr per day for 4 days caused no change in body weight as compared to controls, with the exception of a transient body weight loss on day 1 in the 3 ppm dose group. Organ weights were not altered except on day 7, when weights of spleen, thymus, and kidney were lower in the 3 ppm dose group (Table 1). Histological examination revealed that significant morphological changes were restricted to the respiratory tract. There was incomplete regeneration of the olfactory epithelium in the nasal cavity of mice in the 3 ppm dose group. Also in the high dose, there was prominent fibrosis of the walls of major bronchi with intraluminal fibrosis

Table 3. Granulocyte-macrophage progenitors following MIC exposure.[a]

Exposure group, ppm	Days post exposure	CFU-GM/ femur ($\times 10^2$)
0	5	262 ± 17
1	5	206 ± 5* (21% ↓)
3	5	183 ± 6* (30% ↓)
0	8	223 ± 14
1	8	189 ± 6* (15% ↓)
3	8	158 ± 6* (29% ↓)
0	21	228 ± 10
1	21	232 ± 7 (2% ↑)
3	21	175 ± 5* (23% ↓)

[a] Mean ± SEM, $n = 5$ female B6C3F1 mice.
*$p < 0.01$ vs. controls.

(Fig. 1). In addition, a mild-to-moderate inflammatory response was associated with the bronchi. These changes persisted through a 91-day recovery period.

Bone Marrow

The effect of MIC on bone marrow cellularity is shown in Figure 2. Hypocellularity appeared in the treated groups on day 5 of postexposure, but the results are not significant. On day 8 there was significant dose-related hypocellularity that was present through day 21 in the 3 ppm dose group. The number of pluripotent stem cells (CFU-S) was reduced on day 5 in all dose groups (Table 2). The number of granulocyte-macrophage progenitors (CFU-GM) was significantly reduced on days 5 and 8 in all dose groups, and this depression persisted through day 21 in the 3 ppm dose (Table 3). The effect of MIC on erythroid precursors is also shown in Table 2; a dose-related depression was found on day 8. The erythroid series was not evaluated on days 5 and 21 of postexposure.

Discussion

Hematopoietic cells originate from stem cells that generate progenitor cells programmed to differentiate along specific cell lineages. As these cells differentiate into mature cells with specialized functions, there is a corresponding loss of proliferative capacity. The mechanisms for the regulation of the commitment and proliferation of these progenitors are probably via factors unique to each hematopoietic lineage. Examination of colony formation of the hematopoietic cells following exposure to certain environmental pollutants has proven to be a sensitive indicator of toxicity of various drugs (*13*).

The present study has shown that significant losses of CFU-S, CFU-GM, and CFU-E were found within 1 week following exposure to MIC at both 3 ppm and 1 ppm. At these doses there was damage to the epithelial lining of the respiratory tract. It has been shown that acute exposure of mice to MIC for as short a time period as 2 hr can result in significant pulmonary damage (*14*). The lesion appears to begin as an erosion of the respiratory epithelium, especially of the major bronchi. There is resultant fibrosis and incomplete epithelialization of the major airways. An inflammatory response persists in the major bronchi through 91 days. We attribute the transient bone marrow depression on days 5 and 8 to stress caused by the necrosis of epithelium in the respiratory system. The persistent alteration in CFU-GM on day 21 is probably secondary to the pulmonary damage. In previous studies on the effect of environmental chemicals on the immune system, clear depression of CFU-GMs in mice was usually

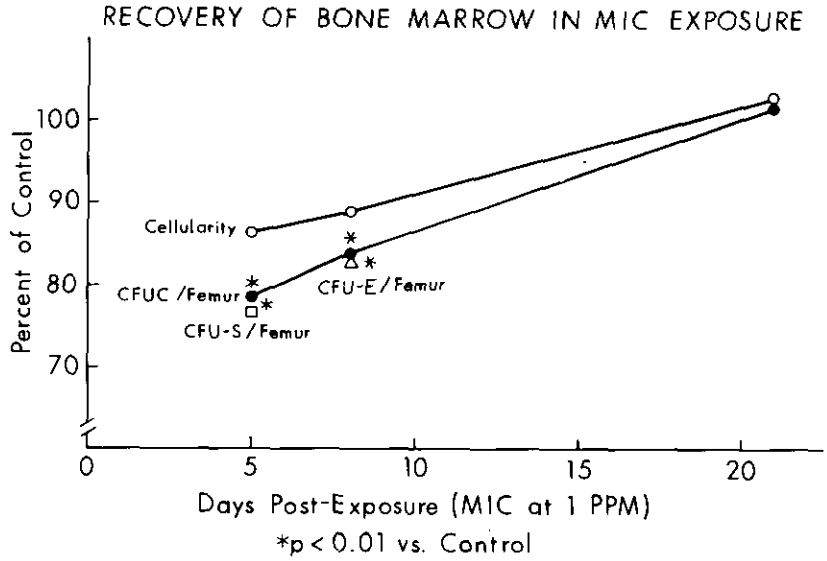

FIGURE 3. *Effect of MIC on bone marrow parameters in the 1 ppm dose group. Colony forming units in culture (CFUC) was expressed as* granulocyte-macrophage progenitors (CFU-GM/10^5 nucleated cells) per femur. The pluripotent stem cells were measured by colony forming units in spleen (CFU-S), and the erythroid precursors were examined by colony forming units in erythrocyte (CFU-E). All were indicated by the percent of controls. (*) denotes significant difference from controls ($p < 0.01$).

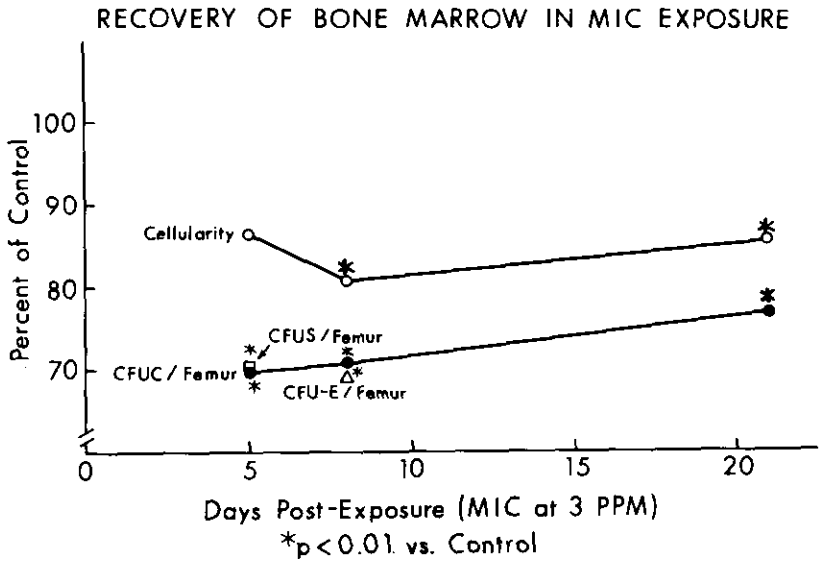

FIGURE 4. Effect of MIC on bone marrow parameters in the 3 ppm dose group. (*) denotes significant difference from controls ($p < 0.01$).

associated with an increased susceptibility to *Listeria monocytogenes* (*15*). The depression in bone marrow progenitors following MIC exposure was not striking at any time after exposure. In companion studies in mice from the same MIC exposure chambers, immune function was assessed using a variety of *in vitro* and host-resistant assays. There were no significant immune alterations, including susceptibility to *Listeria monocytogenes* (*16*). The lack of host resistance effects in this study supports our suggestion that bone marrow effects are secondary and probably not of clinical significance in the mouse. When female mice that had been exposed to 3 or 10 ppm MIC for 2 hr were evaluated 1 year later, there was no difference in bone marrow cellularity nor in granulocyte-macrophage progenitors between control and exposed animals.

The pathogenesis of the early bone marrow depression is not known. The mouse lung is a potent source of stimulating factors for bone marrow cultures *in vitro* (*9,15*), and pulmonary damage could conceivably alter *in vivo* levels of bone marrow stimulating factors. An alternative hypothesis is that the bone marrow depression is mediated through the thymic cortical atrophy that was seen in the MIC-exposed mice. The thymus atrophy was transient (*14*), and functional immunological defects were not found (*16*). However, we have recently reported protection of bone marrow stem cells from the drug-induced suppression by thymic peptides, which were known to influence entry of CFU-S into DNA synthesis (*17*). Furthermore, estrogen-induced alterations in regulatory factors produced by thymic epithelial cells appeared, at least in part, responsible for reduced CFU kinetics (*18*). This suggests that the initial MIC-induced myelotoxicity in mice may be associated at least in part with the role of thymus in bone marrow cell cycle kinetics. In conclusion, MIC exposure appears to cause acute cell death of the lining epithelium of the nasal passages and major airways, and transient alterations of bone marrow parameters that are likely related to pulmonary injury either directly or secondarily through the thymus.

The authors wish to thank Joy Crowson for her excellent secretarial assistance. The authors also thank S. Stefanski and A. Tucker for critical review of the manuscript.

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