Environmental Health Perspectives
Vol. 72, pp. 133-138, 1987

The Toxicity of Inhaled Methyl Isocyanate in F344/N Rats and B6C3F1 Mice. II. Repeated Exposure and Recovery Studies

by John R. Bucher,* Bhola N. Gupta,* Morrow Thompson,* Bernard Adkins, Jr.,† and Bernard A. Schwetz†

F344/N rats and B6C3F1 mice were exposed to 0, 1, 3, or 6 ppm methyl isocyanate by inhalation for 6 hr on 4 consecutive days. Deaths of rats were observed following 3 ppm exposures, and mice died after exposures to 6 ppm. Deaths appeared to be related to severe respiratory distress. Survivors in high dose groups lost weight initially, then gained weight at rates equal to controls throughout a 91-day recovery period. Lung weights increased significantly in male and female rats exposed to 3 ppm, but no persistent changes in brain, kidney, thymus, spleen, liver, or testis weights were seen in either mice or rats.

Blood and serum from male and female rats were taken for clinical pathology and hematology assessments on day 7 of postexposure, the day prior to the first observed deaths of these animals. No changes or only slight changes were seen in measures of serum alanine aminotransferase, sorbitol dehydrogenase, alkaline phosphatase, or in blood and brain cholinesterase activities. However, serum creatine kinase increased with dose in both males and females. Blood urea nitrogen, creatinine, and methemoglobin were unchanged. No changes were seen in counts of red blood cells or platelets, or in red cell indicies. Hemoglobin concentrations and hematocrits were slightly elevated. No changes were noted in absolute leukocyte counts, but counts of segmented neutrophils increased and lymphocytes decreased. These changes are consistent with slight hemoconcentration and a stress-related leukogram, as seen in acute exposure studies. These studies provide toxicity information for further NIEHS studies of immunotoxicity, reproductive toxicity, and genetic toxicity using this repeated exposure regimen, which are also reported in this issue. The results indicate that the respiratory system is the primary site of injury following repeated inhalations of lethal and sublethal concentrations of methyl isocyanate, and give little evidence of direct effects on nonrespiratory tissues.

Introduction

The previous paper in this series outlined changes in F344 rats and B6C3F1 mice following a single 2-hr inhalation exposure to lethal and sublethal concentrations of methyl isocyanate (*1*). This exposure pattern was chosen to mimic in duration and result the December 3, 1984, release of methyl isocyanate from a storage tank at an agricultural chemical plant in Bhopal, India. The results of this study (*1–3*) demonstrated that the lung and upper respiratory tract were the primary targets of methyl isocyanate injury and suggested that damage to the respiratory tract was severe enough to account for the observed mortality. Results of the previous study also demonstrated a very steep dose response for lethality of inhaled methyl isocyanate, with concentrations of 10 ppm causing few or no deaths in rats or mice, but concentrations of 20 to 30 ppm being lethal to 60 to 80% of exposed animals.

Because of the overwhelming pulmonary injury and events secondary to this injury that occur during short exposures to high concentrations of methyl isocyanate, it is possible that direct injury to other organs or organ systems could be obscured using this type of exposure regimen. For this reason we have performed studies which have examined various toxic endpoints in animals exposed repeatedly to lower concentrations of methyl isocyanate by inhalation. In these studies, rats and mice of both sexes were exposed to 0, 1, 3, or in one case, 6 ppm methyl isocyanate for 6 hr on 4 consecutive days. Animals were taken for histopathologic evaluation on postexposure days 7, 28, 49, and 91. Blood was taken from rats for clinical pathology and hematology studies on day 7, and body weights were collected periodically throughout the 3-month postexposure period. This exposure regimen is the same as that used in other NIEHS studies of immunotixicity and myelotoxicity in female mice (*4,5*), and reproductive and genetic toxicity in male and female mice (*6,7*). This report contains data con-

*The National Toxicology Program, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709.
†Northrop Services Inc., P. O. Box 12313, Research Triangle Park, NC 27709.

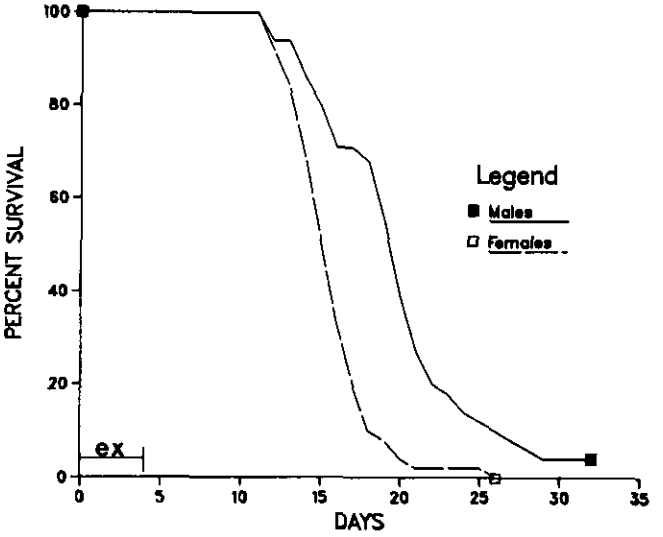

FIGURE 1. Survival of rats following four, 6-hr exposures to 3 ppm MIC, $N = 30$. Actual concentrations were: male rats, 2.98 ± 0.21; female rats, 2.79 ± 0.41 ppm. Exposures occurred during days 1–4 (ex).

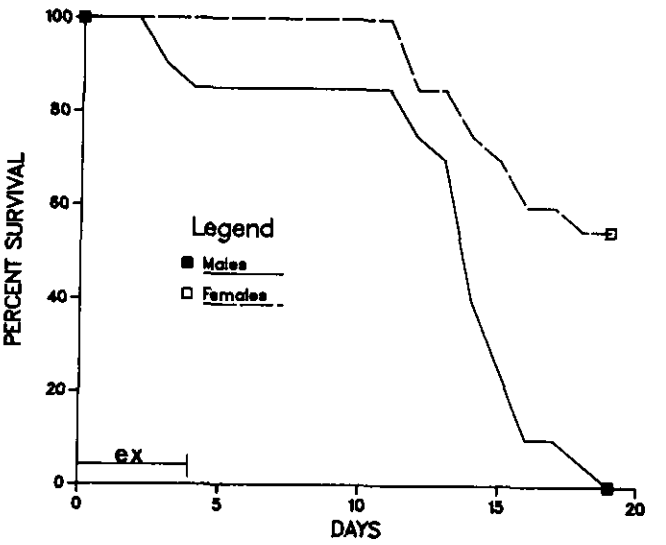

FIGURE 2. Survival of mice following four, 6-hr exposures to 6 ppm MIC, $N = 20$. Actual exposure concentrations were 5.83 ± 1.05 ppm. Exposures occurred during days 1–4 (ex). The experiment was terminated on day 19.

cerning animal mortality, body and organ weights, and results of clinical pathology and hematology studies. Histopathology findings have not yet been finalized, and therefore will be reported elsewhere.

Materials and Methods

Methyl isocyanate (> 99% pure) was supplied by Union Carbide Corp. Male and female F344/N rats and B6C3F1 mice (4–6 weeks old) were obtained from Charles River (Kingston NY, or Portage, MI) and were quarantined for 10 to 21 days prior to random distribution to exposure groups. Exposures were carried out in 1,330 L, stainless-steel exposure chambers. Details of vapor generation, monitoring and safety aspects are reported by Adkins et al. (*8*). Exposures were conducted on 6-17 to 6-20, and 6-25 to 6-28-85. Procedures used, details of animal housing, feed and water, and viral serology and sentinel animal program have been outlined (*1*). Rats used were found negative for antibodies to RCV/SDA, Sendai, KRV, PVM and H-1. Mice were negative for MHV, Sendai, PVM, GDVII and EDIM.

Rats and mice were exposed to concentrations of 0, 1, or 3 ppm MIC for 6 hr on 4 consecutive days. One group of male rats was exposed to 3 ppm for 6 hr on 1 day only, and one group of male and female mice was exposed to 6 ppm, 6 hr per day, for 4 days in range finding studies. On postexposure days 7, 28, 49, and 91, five predesignated animals per group were killed by pentobarbital overdose and subjected to a complete gross necropsy. The lungs, brain, liver, kidney, thymus, testis, and spleen were weighed prior to fixation for histopathologic examination. Immediately prior to necropsy, blood was obtained from the right cardiac ventricle of anesthetized rats on day 7 of postexposure. Each sample was divided, and one fraction was allowed to clot for serum collection; the other fraction was added to a tube containing a premeasured amount of ETDA. Serum samples were analyzed for activities of alanine aminotransferase (*9*), alkaline phosphatase (*10*), creatine kinase (*11*), sorbitol dehydrogenase (*12*), and concentrations of urea nitrogen (*13*), and creatinine (*14*). Samples of whole blood were used for determinations of total blood cell counts (laser optics counter), for preparation of blood smears (morphologic evaluation and differential counts), and for measurements of concentrations of methemoglobin (*15*) and activities of cholinesterase (*16*). Cholinesterase activity was also determined in homogenates of brain tissue. For these analyses, the right hemisphere was taken, chilled, and homogenized in 10 volumes of 0.25 M sucrose. Animals were checked for mortality, morbidity, and clinical signs twice daily throughout the first month, and once daily thereafter.

Statistical Methods

Statistical analyses were performed using the RS/1 Multicompare procedure, the Wilk-Shapiro test for normality, and the pooled variance t test for differences in body and organ weights, clinical pathology, and hematology. Data expressed in the form of ratios were analyzed using the Wilcoxon rank sum test.

Results

Clinical Signs and Mortality

Signs of animal discomfort were generally less intense during the repeated exposures than during single ex-

posures to higher concentrations of MIC (*1*). No nasal or oral discharge was noted during the exposures, but animals exposed to 3 or 6 ppm appeared stressed upon removal from the chambers. Animals were inactive and often had a ruffled haircoat. Rats showed signs of eye irritation and blinking. Signs were most severe after first exposure and lessened during subsequent exposures. Signs of dyspnea were observed in rats exposed to 3 ppm and in mice at 6 ppm at various times during the four exposures (rats were not exposed to 6 ppm in these studies), but breathing was less affected or normal by the start of the next exposure day.

During the first week following the exposures, rats exposed to 3 ppm and mice exposed to 6 ppm showed signs of labored breathing and ruffled haircoat. The signs were similar to those exhibited by animals in the higher dose groups in the single dose studies (*1*) and worsened with time. Three of 20 male mice died during the third and fourth days of exposure to 6 ppm, but most deaths of high dose (3 ppm) rats and high dose mice (6 ppm) began 8 days after the last exposure and continued for several weeks (Figs. 1, 2). These doses killed nearly 100% of male rats, male mice, and female rats, but female mice appeared less affected than the other groups, which is consistent with the findings of the single dose studies (*1*). No male or female rats exposed to 1 ppm, or female mice exposed to 1 or 3 ppm, died during the 91-day follow-up studies; mortality of male mice in 3 ppm was 1/30 in one study, and 1/20 in a second study. No male mice died following exposure to 1 ppm. Severely affected animals rarely ate or drank during periods of respiratory distress. While deaths

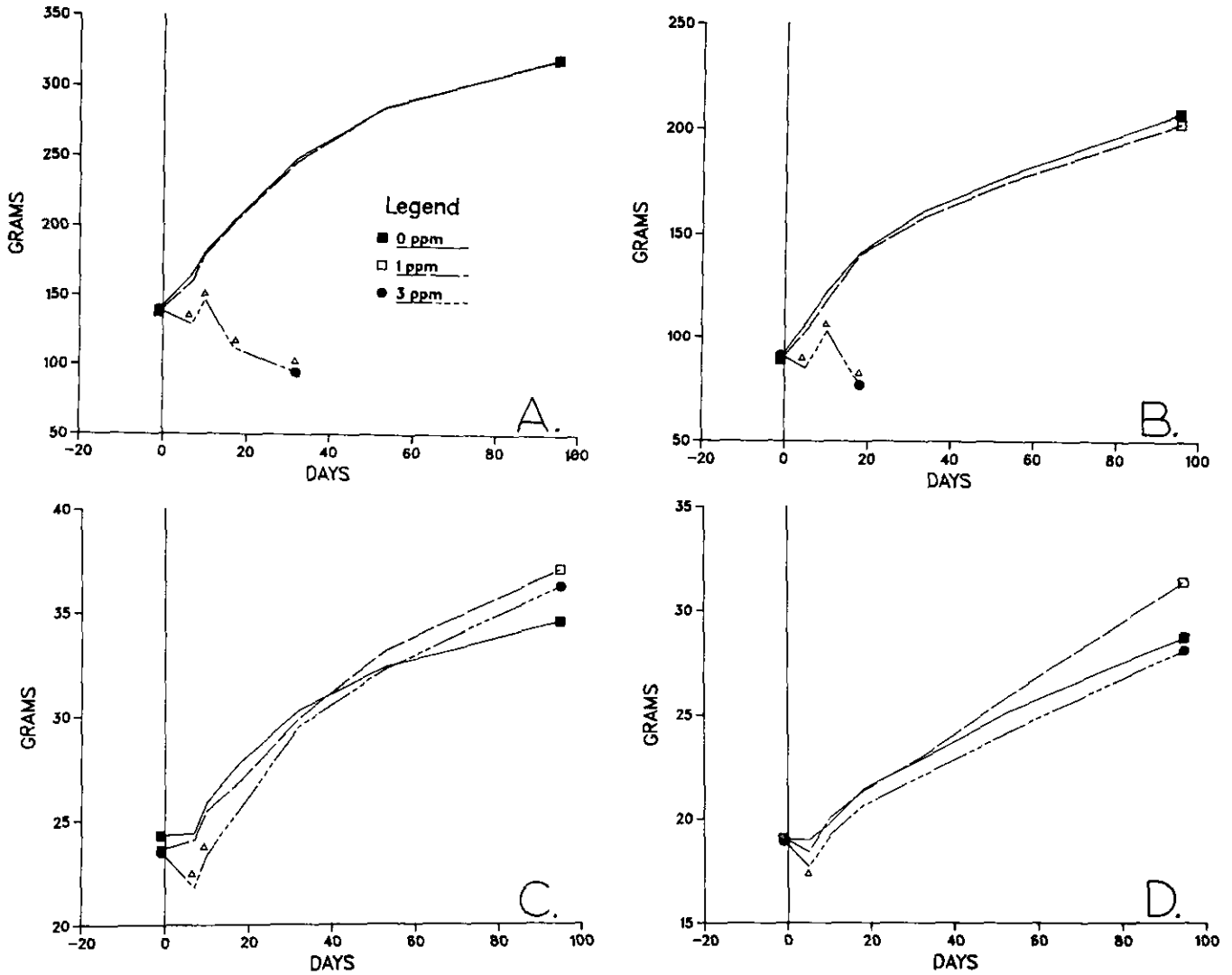

FIGURE 3. Mean body weights for (*A*) male rats, (*B*) female rats, (*C*) male mice, and (*D*) female mice, following exposures to 0, 1, or 3 ppm MIC during days 1–4. *N* began as 20 and decreased by 5 on each postexposure analysis day (7, 28, 49 and 91). Because of deaths, *N* decreased more rapidly for high dose male and female rats. Average chamber concentrations were as follows: male rats and mice, 1.13 ± 0.16, 2.98 ± 0.21; female rats and mice, 1.13 ± 0.23, 2.79 ± 0.41 (△, $p < 0.05$).

were clearly due primarily to respiratory embarrassment, dehydration may have also been a factor.

Body and Organ Weights

Effects on body weights were dose-related in all exposure groups (Fig. 3). Both male and female mice showed an initial weight loss immediately following the exposures to 3 ppm, but weight gain then resumed and achieved rates equal to controls. Both male and female rats showed significant decreases in body weight following the exposures to 3 ppm, and both groups showed a transient gain in weight just prior to the onset of worsening respiratory problems. All high dose female rats died before the postexposure day-28 scheduled sacrifice, and the last two surviving male rats in the high dose group were killed on day 28.

Significant changes in lung weights were seen in male and female rats and in male mice following exposure to 3 ppm MIC (Fig. 4). On days 7 and 28 of postexposure, both the absolute lung weights and lung-to-body weight ratios (not shown) of 3 ppm male rats were significantly greater than in controls. Interestingly, lung weights were even higher in the group of male rats which had been exposed to 3 ppm, 6 hr, for only 1 day (Fig. 4*A*). This group had a body weight that averaged 125 g vs. the control weight of 180 g, 7 days postexposure. Male mice also had a significantly elevated lung-to-body ratio on day 7 postexposure, and absolute lung weights were greater than controls on day 91. No significant increases in lung weight were seen in female mice. Exposure-related changes in lung weights were much less dramatic in mice than in rats during the 91-day postexposure period. However, the severe early changes in rats are consistent with the deaths observed in these groups. The lungs of mice were apparently less affected

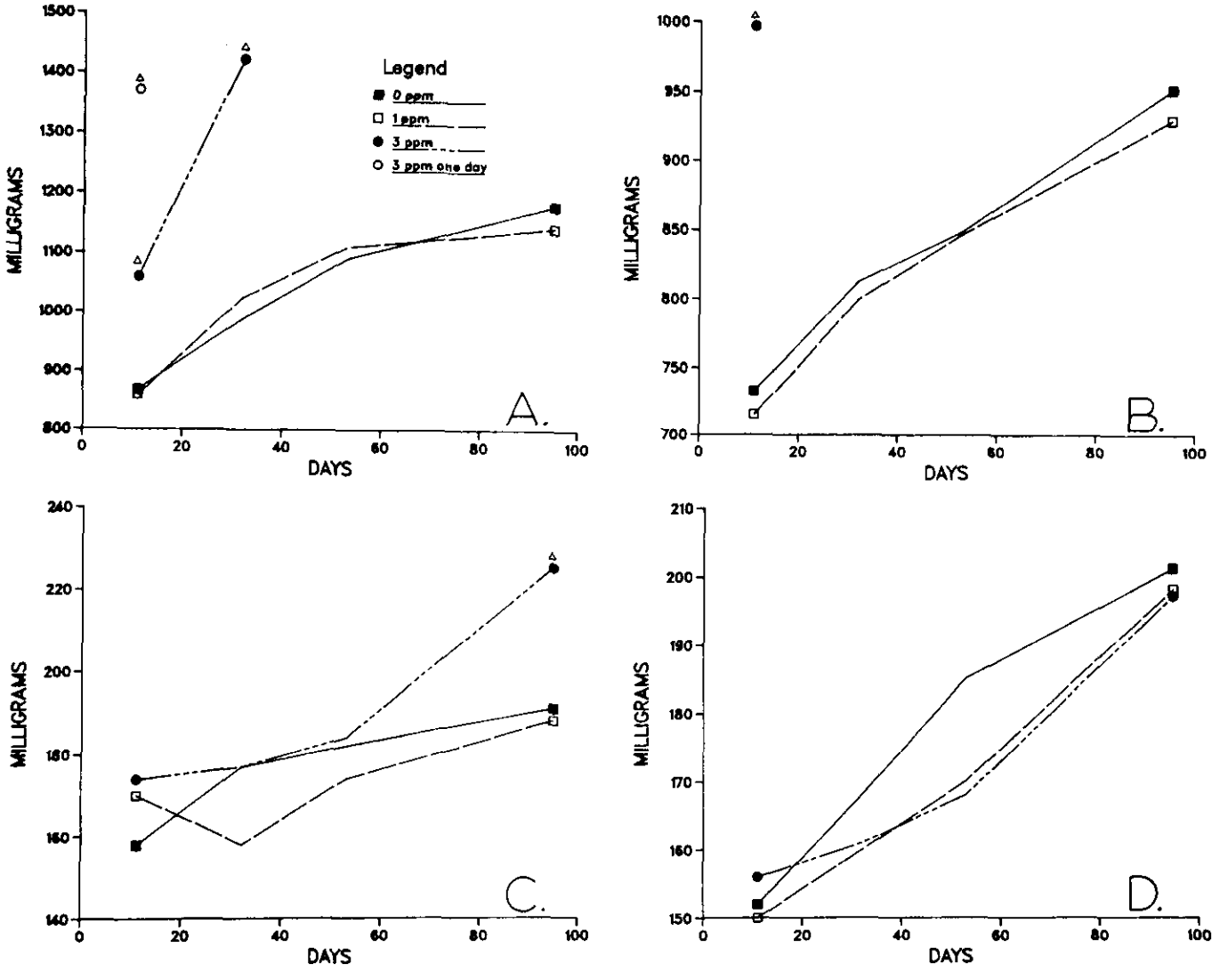

FIGURE 4. Lung weights of (*A*) male rats, (*B*) female rats, (*C*) male mice, (*D*) female mice, following exposures to 0, 1, or 3 ppm for 4 days. The group of male rats indicated by (○) was exposed to 3 ppm for one 6-hr period only. $N = 5$ for each point, except for 3 ppm male rats, day 28, where $N = 2$ (△, $p < 0.05$). Exposures occurred during days 1–4.

by 3 ppm MIC, and consequently mortality was much lower in mice than in rats at 3 ppm.

Thymus-to-body and liver-to-body weight ratios were lower (9 to 16%) in high dose male and female rats than in controls on day 7 postexposure (not shown); excessive mortality prevented analysis of this group later in the study. Spleen-, kidney-, brain- and testis-to-body weight ratios were either unchanged or slightly higher than controls in dosed rats throughout the studies. This may suggest a selective effect on the liver and thymus, but all of these marginal changes are consistent and expected, considering the decrease in body weight and the apparent poor nutritional status of the high dose animals. In mice, absolute thymus weights were lower in high dose (3 ppm) females on day 7 postexposure, but no consistent changes in other organ-to-body weight ratios were seen in any dose group at any time during the study.

Clinical Pathology and Hematology

Sera, blood, and brain tissue were analyzed from male and female rats on day 7 postexposure only. This was the day preceding the onset of deaths of high dose animals. No change or only slight changes in serum activities of alanine aminotransferase, alkaline phosphatase, and sorbitol dehydrogenase were observed in dosed versus control rats, but serum creatine kinase activity was significantly increased in both low and high dose groups of male and female rats (Table 1). Blood urea nitrogen, creatinine and methemoglobin concentrations, and blood and brain cholinesterase activities were unchanged in dosed rats. There were no changes in counts of red blood cells or platelets, or in red cell indices (mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration). Hemoglobin concentrations and hematocrits were mildly increased (< 10%) in high dose male and female rats. Although there were no significant changes in total leukocyte counts, dose-related trends did occur in absolute counts of segmented neutrophils (increased) and lymphocytes (decreased) (Table 2). No changes were noted in absolute counts of other cell types (immature neutrophils, monocytes, eosinophils or basophils).

Table 1. Rat serum creatine kinase activity on postexposure day 7.

Exposure group, ppm	Creatine kinase activity, U/L	
	Males	Females
0	280.4 ± 11.8	235.2 ± 26.8
1	467.8 ± 41.9[a]	425.7 ± 32.7[a]
3	445.8 ± 25.1[a]	604.0 ± 36.8[a]

[a] $p < 0.05$.

Table 2. Total and differential leukocyte counts in rats.[a]

Sex	Exposure group, ppm	Total	Segmented neutrophils	Lymphocytes
Male	0	4.06	0.97	2.93
		(0.63)	(0.20)	(0.53)
	1	3.62	1.00	2.45
		(0.26)	(0.16)	(0.28)
	3	3.78	1.28	2.34
		(0.52)	(0.27)	(0.40)
Female	0	3.14	0.78	2.25
		(0.49)	(0.24)	(0.32)
	1	3.00	0.85	1.98
		(0.50)	(0.28)	(0.29)
	3	3.08	0.97	1.90
		(0.80)	(0.42)	(0.49)

[a] Data are means (SD), 1000/μL, $N = 5$, counts from one outlier omitted from male 3 ppm group.

Discussion

In single dose studies, exposure to MIC at 3 ppm for 2 hr resulted in minimal inflammation in the nasal cavity of rats and mice, and no effects on other indices of toxicity (*1,2*). However, the results of the present study clearly indicate continued exposure to this concentration results in severe injury and death. The sensitivity of the various groups to methyl isocyanate toxicity was similar to that observed in the previous study (*1*), with lethal doses for rats approximately one half those for mice. Female mice appeared less affected than any other group. Male rats exposed for one, 6-hr period to 3 ppm had more respiratory signs following the exposure than did male rats exposed to 3 ppm on 4 consecutive days. All five male rats exposed to 3 ppm for 1 day only were killed on postexposure day 7, so it is not known if this exposure is lethal; however, lung weights and lung-to-body weight ratios were higher than those seen in the group exposed for four, 6-hr periods. High mortality has been observed in all MIC-exposed animals that have shown elevated lung weights, and the degree of increase in lung weight subjectively correlates with the extent of obstructive, fibroproliferative lung changes and the retention of fluid and mucus (*3*). The higher lung weights seen at 7 days of postexposure in male rats exposed for one 6-hr period to 3 ppm than in male rats exposed for 4 days to 3 ppm suggests that fibroproliferative changes may have occurred more rapidly in the lungs of rats exposed for only one 6-hr period to 3 ppm. Preliminary histopathology results support this interpretation.

Exposure to 1 ppm was without apparent effect on the lung or other organs in any group of rats or mice, suggesting that at this concentration, sufficient MIC may not have reached critical regions of the bronchi or bronchioles. MIC may not have reached critical regions if complete scavenging of the highly reactive material (*17*) occurred at higher levels of the respiratory tract. This explanation remains to be confirmed by electron microscopy studies.

Clinical pathology and hematology studies similar to those performed here were conducted on male and female rats immediately after exposure and at various times following single exposures to MIC (*1*). All changes were considered secondary to effects of respiratory injury and to hemoconcentration in dehydrated animals. In the present study, measurements were taken on the day preceding the onset of deaths of rats in the 3 ppm

group. Slight effects of hemoconcentration were again noted in hematologic assessments, and no signs of specific liver or kidney damage were noted in measures of serum alanine aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, and in blood BUN and creatinine. Blood and brain cholinesterase were not affected. The mild changes in absolute counts of segmented neutrophils (increased) and lymphocytes (decreased) were consistent with a stress leukogram, and were similar to changes seen following acute exposures (*1*). Activities of creatine kinase in serum were increased in animals in both the 1 and 3 ppm treatment groups. The increases were mild (approximately 2- to 3-fold) and were not considered to be a significant treatment-related finding. In tissues, creatine kinase activity is high in brain and skeletal and cardiac muscles. High serum activities are generally produced by diseases of or damage to muscular tissue. While a direct effect of MIC or a metabolilte on cardiac or skeletal muscle cannot be ruled out, increases of this magnitude and greater can be produced by physical exercise or exertion, trauma, or muscular cramps. The high dose rats were clearly suffering from respiratory distress. Low dose animals were not, but changes in creatine kinase activity of this magnitude do not suggest that primary muscular injury was a significant component of methyl isocyanate toxicity.

The intent behind the four exposure experimental design was to maximize our ability to detect evidence of direct toxicity of methyl isocyanate to extrapulmonary organs or organ systems. Exposure of rats to 3 ppm and mice to 6 ppm for 4 days resulted in deaths commencing approximately 1 week after the last exposure. All evidence derived from these studies suggests deaths were caused by respiratory compromise, and little evidence for direct, significant extrapulmonary injury was found in measures of organ weights and clinical pathology and hematology in animals exposed repeatedly to lethal or sublethal concentrations of methyl isocyanate by inhalation.

REFERENCES

1. Bucher, J. R., Gupta, B. N., Adkins, B., Jr., Thompson, M., Jameson, C. W., Thigpen, J. E., and Schwetz, B. A. The toxicity of inhaled methyl isocyanate in F344/N rats and B6C3F1 mice. I. Acute exposure and recovery studies. Environ. Health Perspect. 72: 53–61 (1987).
2. Boorman, G. A., Uraih, L. C., Gupta, B. N., and Bucher, J. R. Methyl isocyanate inhalation and 90-day recovery study in B6C3F1 mice. Environ. Health Perspect. 72: 63–69 (1987).
3. Bucher, J. R., Boorman, G. A., Gupta, B. N., Uraih, L. C., Hall, L. B., and Stefanski, S. A. Two-hour methyl isocyanate inhalation exposure and 91-day recovery: a preliminary description of pathologic changes in F344 rats. Environ. Health Perspect. 72: 71–75 (1987).
4. Tucker, A. N., Bucher, J. R., Germolec, D. R., Silver, M. T., Vore, S. J., and Luster, M. I. Immunotoxicity studies on mice exposed to methyl isocyanate. Environ. Health Perspect. 72: 139–141 (1987).
5. Hong, H. L., Bucher, J. R., Canipe, J., and Boorman, G. A. Myelotoxicity induced in mice by methyl isocyanate. Environ. Health Perspect. 72: 143–148 (1987).
6. Schwetz, B. A., Adkins, B., Jr., Harris, M., Moorman, M., and R. Sloane. Reproductive and developmental toxicology studies on inhaled methyl isocyanate. Environ. Health Perspect. 72: 149–152 (1987).
7. Shelby, M. J., Allen, J. W., Caspary, W. J., Haworth, S., Ivett, J., Kligerman, A., Luke, C. A., Mason, J. M., Myhr, B., Tice, R. R., Valencia, R., and Zeiger, C. Results of *in vitro* and *in vivo* genetic toxicity tests of methyl isocyanate. Environ. Health Perspect. 72: 183–187 (1987).
8. Adkins, B., Jr., O'Connor, R. W., and Dement, J. M. Inhalation exposure system used for acute and repeated-dose methyl isocyanate exposures of laboratory animals. Environ. Health Perspect. 72: 45–51 (1987).
9. Wroblewski, F., and LaDue, J. S. Serum glutamic-pyruvic transaminase in cardiac and hepatic disease. Proc. Soc. Exp. Biol. Med. 91: 569–571 (1956).
10. Bowers, G. N., and McComb, R. D. Measurement of total alkaline phosphatase activity in human serum. Clin. Chem. 22: 650–656 (1975).
11. Szasz, G. Gruber, W., and Bernt, E. Creatine kinase in serum. I. Determination of optimum reaction conditions. Clin. Chem. 22: 650–656 (1976).
12. Asada, M., and Galambos, J. T. Sorbitol dehydrogenase and hepatocellular injury: an experimental and clinical study. Gastroenterology 44: 578 (1963).
13. Talke, H., and Schubert, G. E., Enzymatische harnstoffbestimmung in blut und serum in optischen test nach Warburg. Klin. Wochenschr. 43: 174–175 (1965).
14. Helger, R., Rindfrey, H., and Hilgenfeldt, J., Eine methode zur direkten bestimmung des creatinins in serum und harn ohne enteiweissung nach einer modifizierten Jaffe-method. Z. Klin. Chem. Klin. Biochem. 12: 344 (1974).
15. Evelyn, K. A., and Malloy, H. T. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. J. Biol. Chem. 8: 655–662 (1938).
16. Ellman, G. L., Cortney, K. D., Andres, V., and Fetherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88–95 (1961).
17. Union Carbide Corp. Methyl Isocyanate, F41443A. Union Carbide Corporation, Danbury, CT, 1976.