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Methyl Isocyanate Eight-Day Vapor Inhalation Study with Fischer 344 Rats

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Groups of ten male and ten female Fischer 344 rats were exposed by inhalation3.1, 0.6, 0.15, or 0.0 (control) ppm of methyl isocyanate (MIC) vapor 6 hr per day for 8 days (two 4-day sessions separated by a 2-day rest). Evaluation of toxic effects included body weight, food consumption, organ weights, and selected hematologic, ophthalmic, neurologic, gross anatomic, and histologic examinations. There were no deaths during the study. Rats of the 3.1 ppm exposure group had decreased body weights, food consumption, and blood oxygen saturation (males only). An increase in hemoglobin concentration (males only) and in lung weights (absolute and as a percentage of body weight) were also observed in the 3.1 ppm rats. Ophthalmic or neurofunctional behavior evaluations were negative for all MIC exposure groups. Only 3.1 ppm of MIC vapor resulted in lesions in the respiratory tract, 0.6 or 0.15 ppm did not. The types of lesions observed were inflammation and squamous metaplasia in the nasal cavity, trachea, and bronchi; inflammation of the bronchioles and alveoli; and submucosal fibroplasia of the bronchioles. No significant lesions were observed in tissues other than those of the respiratory tract in all MIC exposure groups. The results of this study indicate the current 0.02 ppm threshold limit value for MIC is not too high regarding toxicity.

Introduction

Our previous acute inhalation studies with methyl isocyanate (MIC) vapor determined the LC_{50} values and described the type and severity of respiratory tract lesions observed in Fischer 344 rats, B6C3F1 mice, and Hartley guinea pigs (*1–3*). The types of biological responses observed in these acute studies confirmed the potent irritancy and suspected tissue reactivity of MIC vapor. Furthermore, each species appeared to be a suitable model for assessment of MIC irritancy and toxicity, although a few differences were observed in the respiratory tract following microscopic evaluation (*2,3*).

The objective of the present study, initiated in 1980 (prior to the Bhopal tragedy), was to examine the toxic manifestations in rats following 8-day repeated exposure to MIC vapor. As the 6-hr LC_{50} value for Fischer 344 rats was 6.1 ppm MIC (*1*), the target MIC concentrations selected for the repeated (8-day) exposure study were 3.0, 0.6, 0.12, and 0.0 (control) ppm. A second repeated exposure study was conducted to describe more fully the lesions in the rat respiratory tract as a result of either 4 or 8 days of exposure to MIC at similar exposure concentrations and to characterize subsequent development of lesions during a 3-month recovery period (*4,5*).

The current 8-hr threshold limit value (TLV) for MIC of 0.02 ppm (*6*) was established because of its mucous membrane irritation and sensitization potential. The present investigation was performed at MIC concentrations ranging from 8 to 150 times the current TLV.

Materials and Methods

Details of the materials, vapor generation methods, and analytical procedures used in this study have been described elsewhere (*4*). Briefly, vapors of MIC-nitrogen gas were metered from a stainless-steel cylinder (*1*) containing liquid MIC into stainless-steel and glass chambers (4350 L), operated at airflows of 1050 to 3000 L/min to give the desired target chamber concentration. Chamber air was analyzed for MIC one to three times an hour with a Hewlett Packard 5710A gas chromatograph equipped with a nitrogen-phosphorus detector. Chamber air samples of 50 μL to 5 mL, depending on the target chamber concentration, were taken with Pressure Lok syringes. Calibration of the gas chromatograph was performed with liquid injections of MIC in *n*-hexane standard solutions prepared volumetrically. The minimum detection limit was approximately 50 ppb.

Animal Species, Source, and Husbandry

Male and female Fischer 344 rats (COBS CDF (F344)/CrlBR), 33 days of age, were received from Charles River Breeding Laboratories (Portage, MI). Upon ar-

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rival, fecal samples were examined for intestinal parasites by zinc sulfate flotation. The results were negative. The animals were kept on a 12-hr light/dark photoperiod throughout the study. Water, supplied by an automatic watering system, and powdered NIH-07 feed (Batch #553) supplied by Zeigler Brothers, Inc. (Gardners, PA) were available *ad libitum* except during inhalation exposures. The rats were separated into exposure groups and sex, numbered by toe clipping, and housed two per cage in stainless-steel, wire-mesh cages. A layer of Deotized Animal Cage Board (Upjohn Company, Kalamazoo, MI) was placed under each shelf of cages except during inhalation exposures. For the inhalation exposures, rats were transferred from their home cages to stainless-steel, wire-mesh cages (2 rats/cage) in the chamber.

Body weight and physical condition of all rats were followed for approximately 2 weeks prior to randomized assignment into exposure groups of ten per sex. At the time of group assignment, only animals with body weights within two standard deviations of the group mean for each sex were used in the study. Any animal in poor health or having eye lesions was rejected from group assignments.

Target Concentrations and Exposure Regimen

Target concentrations of 3.0, 0.6, and 0.12 ppm were selected for this study. Animals (60 days old) were exposed to MIC vapor, 6 hr per day for 4 consecutive days. After 2 days of nonexposure (weekend period) animals were exposed for an additional 4 consecutive days. Control (air-exposed) animals were handled in an identical manner as MIC-treated animals. To compensate for any possible undetected variation in chamber exposure conditions (e.g., concentration, temperature, relative humidity), the position of rat cages was rotated within each chamber prior to exposure.

Biological Evaluations

Clinical inspection of the rats was performed daily. A modified Irwin screen (*7*) was performed on five rats per sex of the 3.0, 0.6, and 0.0 ppm groups just prior to the first MIC exposure and immediately following the eighth MIC exposure. All rats of the 3.0 and 0.0 ppm groups had a gross ophthalmic examination prior to initiation of the exposure regimen and just prior to sacrifice. A light and magnifying lens was used to observe the cornea and anterior chamber of the eye. Body weights were determined on the morning preceding the first, second, fourth, and sixth exposure and again immediately prior to sacrifice. Food consumption was measured for an approximate 15-hr period following the third and eighth exposure. Rats were killed by exsanguination the morning following the last exposure by severing the brachial blood vessels following anesthesia with methoxyflurane. Rats were subjected to a complete necropsy examination which involved a thorough external examination followed by the opening of all body cavities, as well as the cranium, and examination of the viscera. The following tissues were placed in 10% neutral buffered formalin (NBF): entire respiratory tract, bronchial lymph nodes, kidneys, liver, testes, and gross lesions. The lungs were gently infused and inflated with 10% NBF via the trachea, and the nasal passages were flushed with 10% NBF before these tissues were placed in fixative. Tissues were examined microscopically from all rats of the high concentration and control groups. In addition, the respiratory tracts of rats of the intermediate and low concentration groups were examined microscopically. The lungs, liver, kidneys, and testes were weighed at the time of sacrifice. Just prior to severing the brachial blood vessels, blood was obtained from the orbital sinus of each rat. Blood collection tubes contained EDTA as an anticoagulant. The hematologic assessments were hemoglobin, oxygen saturation, carbon monoxide saturation, and erythrocyte fragility. An OSM_2 Hemoximeter® spectrophotometer was used to measure hemoglobin, oxygen saturation, and carbon monoxide saturation. Sodium dithionite was used to reduce the blood sample (i.e., eliminate oxygen) for carbon monoxide determination. For erythrocyte fragility, the minimum and maximum concentration of phosphate-buffered saline causing hemolysis was determined.

Statistical Analysis

Results of the quantitative continuous variables such as body weight changes were intercompared among the MIC test groups and the control group using the following tests: Bartlett's homogeneity of variance (*8*), analysis of variance (ANOVA), and Duncan's multiple range (*9–11*). The latter was used when the F value from the ANOVA was significant. When Bartlett's test indicated heterogeneous variances, the F-test (*8*) was used to compare each group versus the control. When these individual F-tests were not significant, Student's t-test (*8*) was used; when F-tests were significant, the means were compared by the Cochran t-test (*12*). Discontinuous data were compared using Fisher's exact test. A fiducial limit of 0.05 (two-tailed) was used as a critical level of significance.

Table 1. Chamber concentrations of methyl isocyanate during the 8-day vapor inhalation study.

	Target concentrations, ppm			
	0.0	0.12	0.6	3.0
Exposure day	Daily mean chamber concentrations,[a] ppm			
1	0.0 ± 0.0	0.17 ± 0.02	0.50 ± 0.07	3.8 ± 0.6
2	0.0 ± 0.0	0.19 ± 0.05	0.76 ± 0.21	3.9 ± 0.4
3	0.0 ± 0.0	0.15 ± 0.02	0.63 ± 0.10	2.8 ± 0.4
4	0.0 ± 0.0	0.14 ± 0.05	0.52 ± 0.02	2.6 ± 0.1
5	0.0 ± 0.0	0.17 ± 0.04	0.60 ± 0.05	3.0 ± 0.2
6	0.0 ± 0.0	0.12 ± 0.01	0.54 ± 0.02	2.9 ± 0.1
7	0.0 ± 0.0	0.11 ± 0.06	0.55 ± 0.03	2.8 ± 0.1
8	0.0 ± 0.0	0.12 ± 0.01	0.52 ± 0.03	2.8 ± 0.1
Mean of daily means	0.0 ± 0.0	0.15 ± 0.03	0.58 ± 0.09	3.1 ± 0.5

[a] Values represent mean ± SD.

Results and Discussion

Chamber Concentrations and Environmental Conditions

The chamber analytical concentrations of MIC for the 8 days of exposure are presented in Table 1. The mean analytical concentrations were 3.1, 0.6, and 0.15 ppm for target concentrations of 3.0, 0.6, and 0.12 ppm, respectively. The MIC concentration range during each 6-hr exposure was very narrow, as indicated by the small standard deviation of each daily mean. Nominal chamber concentrations were not calculated because the concentration of the N_2-MIC mixture in the head space of the generation cylinder was not determined. Daily mean chamber temperature and relative humidity for all exposure groups ranged from 19.3 to 23.9°C and 50 to 62%, respectively. The housing quarters for the animals were within a temperature range of 19 to 24°C and had a relative humidity range of 46 to 61% throughout the study period.

Mortality, Clinical Observations, Ophthalmic Evaluations, and Neurofunctional Behavior

No animals died during the study. Male and female rats exposed to 3.1 ppm of MIC had a higher incidence of a reddish crust or clear liquid nasal discharge than air-exposed controls during the exposure regimen. These rats also appeared unkempt, and audible respiration was noticed, particularly in the males. On the day of sacrifice, no eye abnormalities were observed in the 3.1 ppm or control rats.

Results from the Irwin screen indicated no abnormal behavior in male or female rats of any MIC exposure group when tested on exposure days 1 and 4. Following the final exposure day, all five of the male and female rats of the 3.1 ppm group exhibited impaired gaits and arched backs. No abnormalities were observed in rats from the 0.6 ppm group. The arched backs were presumably due to respiratory difficulties, while the ataxia was attributed to muscular weakness and postural changes resulting from the large loss in body weight which occurred in these rats.

Body Weights and Food Consumption

Male and female rats of the 3.1 ppm group lost a significant amount of body weight compared to controls after the first exposure (Table 2). In both sexes, the decrease continued with additional MIC exposures. Both males and females of the 3.1 ppm group gained some weight during the 2-day nonexposure period, although their body weight changes remained lower than control values. Male rats exposed to 0.6 ppm MIC had a significant ($p < 0.05$) gain in body weight after one exposure; however, this finding does not appear to be of biological importance. Throughout the remaining exposure regimen, rats of the 0.6 or 0.15 ppm groups did not show differences in body weight gain compared to controls.

The mean food consumption values for the male and female rats of the 3.1 ppm test group were significantly lower than the values for the control group following exposure days 3 and 8 (Table 3). Except for a few isolated instances, no further significant differences were found in the remaining MIC test groups.

Blood Analysis

Exposure to MIC had no effect on red blood cell fragility. The male rats of the 3.1 ppm exposure group had a statistically significant increase in hemoglobin concentration and a decrease in oxygen saturation compared to controls (Fig. 1). No other statistically significant differences were found among groups of male or female rats in the remaining hematologic parameters assessed. These hematologic alterations appear to be compensatory responses. For example, the decreased oxygen content of hemoglobin was probably related to an impairment of gas exchange function of the lung (*13*). Mucous plugs were observed microscopically in the bronchioles of these rats, suggesting heterogeneity of

Table 2. Body weight change for male and female Fischer 344 rats during the methyl isocyanate 8-day vapor inhalation study.

Sex	Mean chamber concentrations, ppm	Initial body weight, g[a]	Mean change from initial body weight,[a] g — Completed exposure days 1	3	5	8
Male[b]	3.1	218.64 ± 10.42	−26.53* ± 3.60	−32.85* ± 4.16	−18.81* ± 5.92	−40.05* ± 7.67
	0.6	222.03 ± 8.95	3.91† ± 1.68	8.02 ± 1.32	17.09 ± 1.42	25.50 ± 2.59
	0.15	219.21 ± 11.25	1.77 ± 1.15	7.55 ± 2.43	16.42 ± 2.94	24.25 ± 3.67
	0	221.31 ± 9.10	1.83 ± 2.00	8.28 ± 2.21	17.14 ± 2.97	25.77 ± 3.69
Female[b]	3.1	142.51 ± 7.80	−17.50* ± 2.29	−17.46* ± 3.66	−5.34 ± 4.79	−24.52* ± 10.87
	0.6	145.31 ± 4.09	−1.13 ± 3.76	3.93 ± 1.32	13.37 ± 1.81	13.29 ± 2.83
	0.15	143.63 ± 5.40	0.88 ± 4.00	4.99 ± 3.22	14.40 ± 4.22	13.59 ± 2.81
	0	145.44 ± 5.88	−0.54 ± 2.50	3.29 ± 3.39	11.94 ± 2.71	12.57 ± 3.19

[a] Values represent mean ± SD.
[b] $N = 10$ rats/group.
* $p < 0.001$ compared to controls.
† $p < 0.05$ compared to controls.

Table 3. Mean food consumption for male and female Fischer 344 rats during the methyl isocyanate 8-day vapor inhalation study.

Sex	Mean chamber concentrations, ppm	Mean food consumption,[a] g/rat/day: Days before exposure 13	Days before exposure 12	Completed exposures 3	Completed exposures 8
Male[b]	3.1	16.52 ± 2.55	16.07 ± 0.54	7.48† ± 0.68	4.98† ± 2.39
	0.6	15.39 ± 0.72	15.87 ± 1.09	19.85 ± 5.58	15.34 ± 0.26
	0.15	16.84* ± 0.91	16.84 ± 0.80	19.21 ± 0.68	16.01 ± 0.72
	0	15.19 ± 0.58	15.98 ± 1.33	19.85 ± 1.86	16.10 ± 0.88
Female[b]	3.1	11.59 ± 0.73	11.28 ± 0.72	6.22† ± 0.84	4.72† ± 2.18
	0.6	11.69 ± 0.92	11.16 ± 0.34	13.63 ± 0.59	12.48 ± 0.70
	0.15	11.93 ± 0.63	10.82* ± 0.33	13.03 ± 1.15	11.59 ± 0.72
	0	12.46 ± 1.01	11.94 ± 0.73	13.31 ± 0.93	11.84 ± 0.75

[a] Values represent mean ± SD.
[b] $N = 10$ rats/group.
* $p < 0.01$ compared to controls.
† $p < 0.001$ compared to controls.

Table 4. Microscopic findings in the respiratory tract of Fischer 344 rats at the conclusion of the methyl isocyanate 8-day study

Organs/lesions	MIC concentration, ppm: 3.1 Males	3.1 Females	0.6 Males	0.6 Females	0.15 Males	0.15 Females	0.0 Males	0.0 Females
Nasal passages								
Number of rats examined	10	10	10	10	10	10	10	10
Rhinitis	9*	10*	1	2	0	2	1	1
Epithelial degeneration/necrosis	4	0	0	0	0	0	0	0
Squamous metaplasia	10*	10*	0	0	0	0	0	0
Trachea								
Number of rats examined	10	10	10	10	10	10	10	10
Tracheitis	9*	6*	0	0	0	0	0	0
Ulceration	1	0	0	0	0	0	0	0
Squamous metaplasia	10*	9*	0	0	0	0	0	0
Regenerative hyperplasia	1	1	0	0	0	0	0	0
Lungs/bronchioles								
Number of rats examined	10	10	10	10	10	10	10	10
Bronchiolitis	10*	9*	0	0	0	0	0	0
Pneumonitis	5*	7*	0	0	0	0	0	0
Squamous metaplasia	4	9*	0	0	0	0	0	0
Regenerative hyperplasia	0	1	0	0	0	0	0	0
Intraluminal and submucosal fibroplasia	4	0	0	0	0	0	0	0
Bronchial lymph nodes								
Number of rats examined	8	7	5	9	7	7	7	6
Reactive hyperplasia	5*	5*	0	0	0	0	0	0

* $p < 0.05$ compared to controls.

lung ventilation. Thus, a decrease in blood oxygen content may explain the decrease in oxygen content of hemoglobin, and the increase in hemoglobin concentration may have resulted from a diminished hemoglobin oxygen saturation. Determination of hematologic parameters in the present study was a result of a suspected interaction between MIC and hemoglobin. Carbamylation of either sodium or potassium isocyanate with hemoglobin has been reported (*14,15*). Furthermore, Lee (*16*) observed a carbamylation reaction *in vitro* between liquid MIC and hemoglobin S from sickle cell patients. Subsequent to this study, experiments were performed to determine the influence of MIC on oxygen-binding properties of guinea pig blood (*17*). Although no data are available regarding the metabolism of MIC, the absence of carbon monoxide saturation indicates, indirectly, that MIC is not largely metabolized to carbon monoxide *in vivo*.

Organ Weights

Absolute lung weights increased 28 and 49% over control animal values for the 3.1 ppm male and female rats, respectively (Fig. 2). Relative lung weights (a percentage of body weight) of the 3.1 ppm rats were also significantly increased from the control mean values. The mean absolute weights of the liver, kidneys, and testes were decreased in rats of the 3.1 ppm group, but

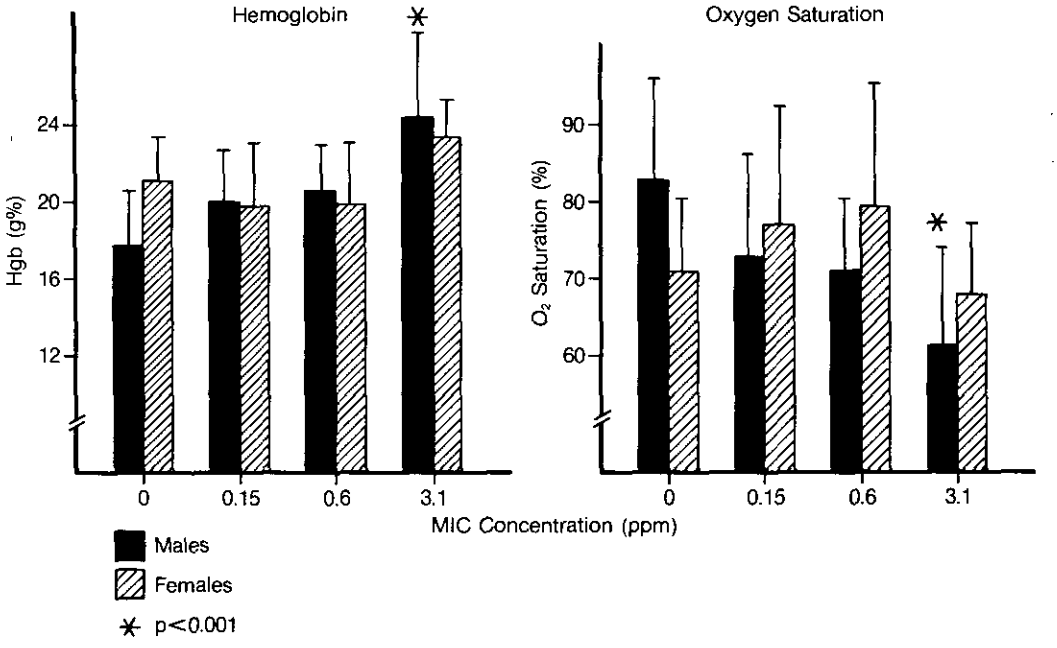

FIGURE 1. Hemoglobin and oxygen saturation results for male and female Fischer 344 rats at the conclusion of the 8-day methyl isocyanate exposure regimen. Group size was $N = 10$ for both sexes. The mean and SD are depicted by each bar.

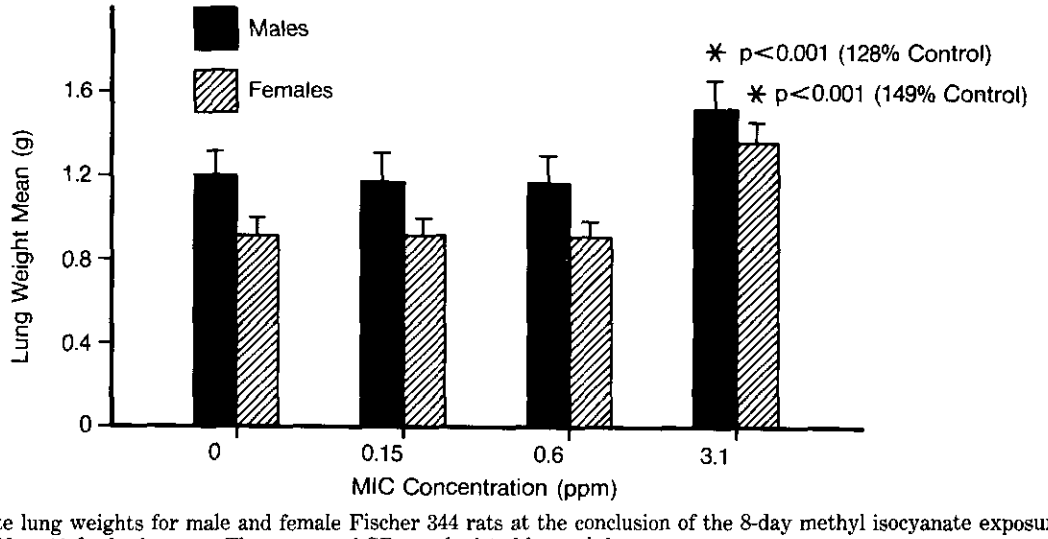

FIGURE 2. Absolute lung weights for male and female Fischer 344 rats at the conclusion of the 8-day methyl isocyanate exposure regimen. Group size was $N = 10$ for both sexes. The mean and SD are depicted by each bar.

in the absence of gross and microscopic lesions, these alterations were considered to be a reflection of the significant body weight losses which occurred in these animals. For rats of the 0.6 and 0.15 ppm groups, there were no differences in mean absolute organ weights when compared to respective control animal values.

Histopathology

Gross lesions of biological significance were confined to the lungs of male and female rats exposed to 3.1 ppm of MIC vapor and consisted of reddening of the lungs (pulmonary congestion) that was distributed over the surface in either a patchy (most frequent), focal, or generalized fashion. Microscopic findings of biological significance were also confined to the respiratory tract of the 3.1 ppm-exposed male and female rats, and consisted of inflammation, epithelial degeneration, squamous metaplasia, and regenerative hyperplasia extending down to and including the respiratory bronchioles. Table 4 presents the distribution of lesions found in the respiratory tract. Rhinitis was present in both sexes, with slightly more severe rhinitis occurring in the males. Rhinitis was moderate or marked in most in-

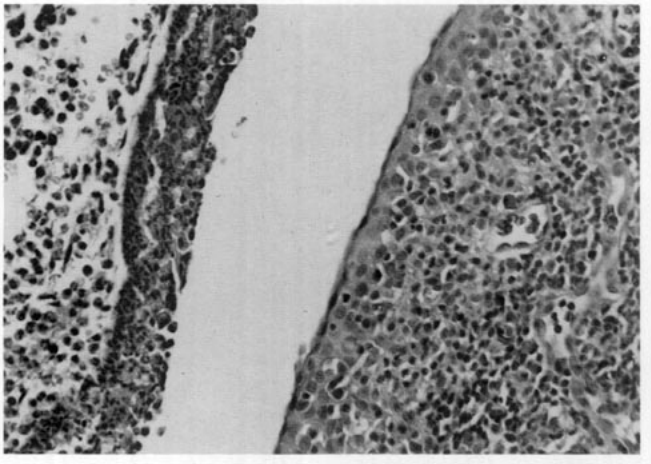

FIGURE 3. Chronic rhinitis with purulent exudate in the anterior nasal cavity of a male rat exposed to 3.1 ppm of methyl isocyanate vapor for 8 days. H & E, × 250. (Reprinted courtesy of Academic Press.)

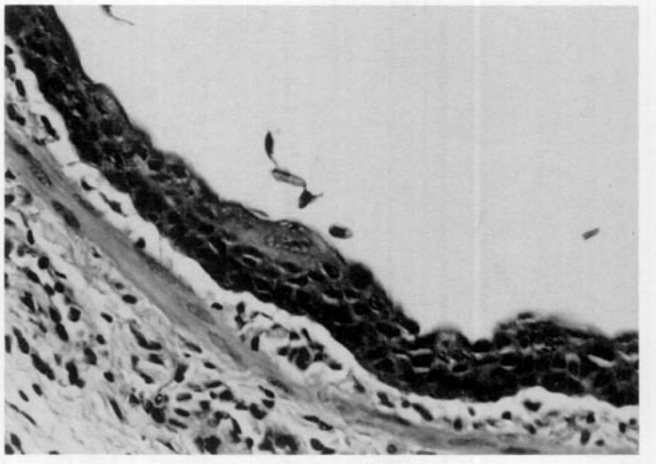

FIGURE 5. Squamous metaplasia of the bronchiolar epithelium of a male rat exposed to 3.1 ppm of methyl isocyanate vapor for 8 days. H & E, × 250. (Reprinted courtesy of Academic Press.)

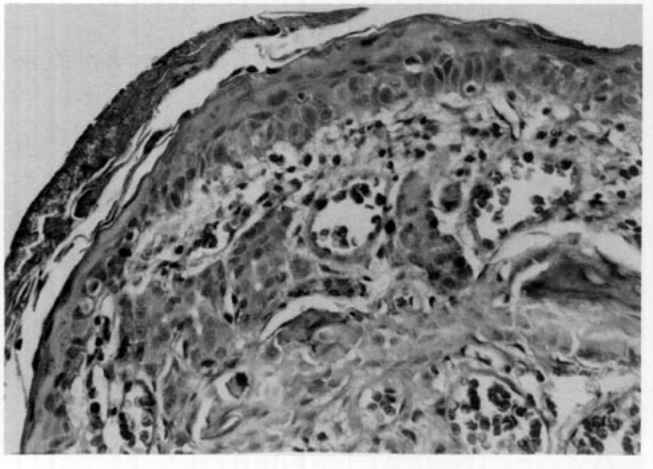

FIGURE 4. Squamous metaplasia of turbinate mucosa in the anterior nasal passage of a male rat exposed to 3.1 ppm of methyl isocyanate vapor for 8 days. H & E, × 250. (Reprinted courtesy of Academic Press.)

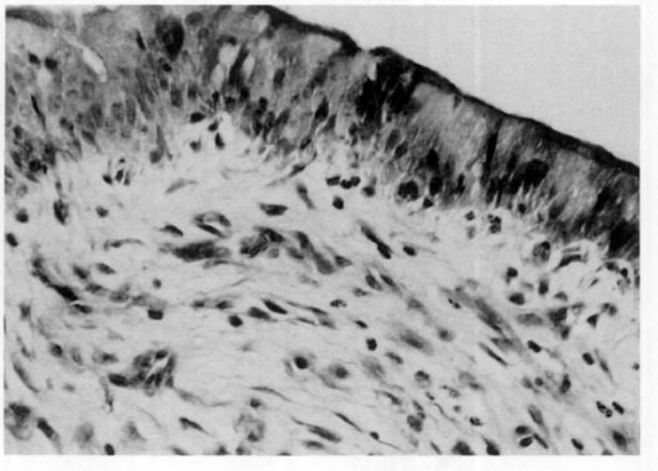

FIGURE 6. Immature submucosal fibroplasia covered by regenerative epithelium in an intraluminal polyp extending from the bronchiolar wall in a male rat exposed to 3.1 ppm of methyl isocyanate vapor for 8 days. H & E, × 250. (Reprinted courtesy of Academic Press.)

stances (Fig. 3). Squamous metaplasia of moderate or marked severity was seen in both sexes in the respiratory mucosa of the nasal cavity (Fig. 4) and extended throughout the respiratory tract down into the bronchioles (Fig. 5). Epithelial cell degeneration was present in both the respiratory and olfactory mucosa of the nasal cavity in the males.

A biologically important lesion found primarily in the lungs of male rats exposed to 3.1 ppm was submucosal fibroplasia. These lesions developed in the walls of the bronchioles and projected into the lumina of the airways as polyps. These polypoid projections were covered by regenerative epithelium and contained cores of immature connective tissues (Fig. 6). Some of the lesions were quite large, although there was no microscopic evidence that they obstructed the airway. It is believed that these focal or multifocal areas of submucosal fibroplasia were in response to ulceration of overlying epithelium following necrosis induced by the MIC vapor. Similar lesions have been reported in rats exposed to toluene diisocyanate (TDI) and were termed bronchiolitis fibrosa obliterans *(18,19)*.

Reactive hyperplasia of the bronchial lymph nodes was present in both the male and female rats of the 3.1 ppm exposure group. Bronchiolitis was present in both male and female rats exposed to 3.1 ppm of MIC vapor, but was more severe in male rats. Mucous plugs containing inflammatory cells were seen frequently in the bronchioles of both sexes and were believed to have originated farther up in the respiratory passages and to have been inspired to the point of lodgement (Fig. 7).

To describe more fully the lesions in the rat respiratory tract as a result of either 4 or 8 days of exposure

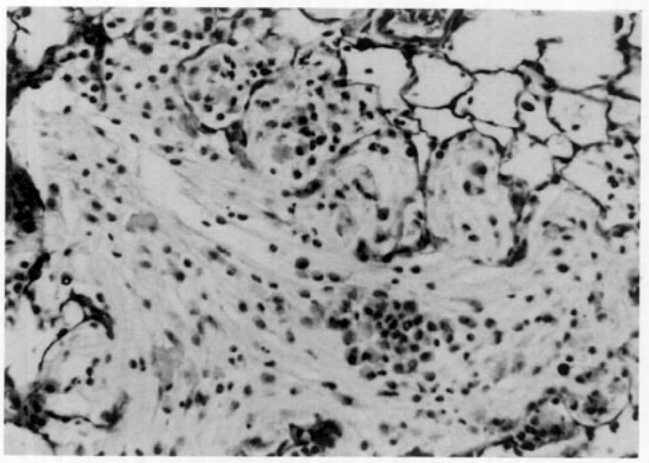

FIGURE 7. Inspired mucus and inflammatory cells in the terminal bronchiole, alveolar duct, and alveoli of a male rat exposed to 3.1 ppm of methyl isocyanate vapor for 8 days. H & E, × 160. (Reprinted courtesy of Academic Press.)

to 3 ppm of MIC and to characterize the subsequent development of these lesions during a 3-month recovery period, an additional repeated exposure study was performed (*4,5*). Recovery from the necrotizing and irritating effects of MIC vapor was observed.

Conclusion

Previous inhalation studies with MIC examined the biological responses which occurred following a single 4- or 6-hr exposure (*1,2,3,20*). The present study focused on the biological responses which resulted from repeated exposures of MIC. As expected from the acute studies, signs of irritancy on the mucosal surfaces of the eyes, nose, and mouth were observed following repeated exposure of 3.1 ppm, and the location of MIC-induced morphologic alterations was restricted to the respiratory tract. In human volunteer studies, irritation of the eyes, nose, and throat were perceived at 0.5 ppm of MIC vapor for short durations (*21*). A concentration of 0.4 ppm (*20*) or 0.3 ppm (*22*) produced no irritation. The current TLV for MIC is 0.02 ppm (*6*) due to mucous membrane irritation and sensitization potential. The results of the present investigation indicate the current TLV is not too high regarding toxicity, since no adverse effects were observed in rats from repeated exposure of concentrations approximately 30 times greater than 0.02 ppm. Other investigators working with laboratory animal models to quantitate the stresses of sensory and pulmonary irritation agree with the current TLV for MIC (*23*).

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