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ORIGINAL INVESTIGATION

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Comparative toxicity of methyl isocyanate and its hydrolytic derivatives in rats

II Pulmonary histopathology in the subacute and chronic phases

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Abstract This paper describes the long-term (subacute and chronic) histopathological effects in the lungs of rats subjected to a single exposure to methyl isocyanate (MIC) by both the inhalation and subcutaneous (s.c.) routes as well as the role of methylamine (MA) and *N,N'*-dimethylurea (DMU), the hydrolytic derivatives of MIC in eliciting the observed changes. At the subacute phase, the intra-alveolar and interstitial edema were prominent only in the inhalation group as against the more pronounced inflammatory response in the s.c. route. With the progress of time the evolution of lesions appeared to be similar, culminating in the development of significant interstitial pneumonitis and fibrosis. MA, one of the hydrolytic derivatives of MIC, also caused interstitial pneumonitis progressing to fibrosis, albeit to a lesser extent than MIC, indicating its contribution to the long-term pulmonary damage. The diffuse interstitial pulmonary fibrosis observed at 10 weeks after a single exposure to MIC by either route is of greater significance in the context of the occurrence of pulmonary fibrosis in the late autopsies of Bhopal gas victims and also clinical sequelae in some of the survivors.

Key words Methyl isocyanate toxicity
Methylamine toxicity · Pulmonary pathology
Long-term effects · Fibrosis · Rats

Introduction

Inhalation of methyl isocyanate (MIC) affects primarily the lungs, the target organ which bore the brunt of lesions in the Bhopal gas disaster. The autopsy findings in the immediate and early deaths revealed extensive damage to the upper respiratory airways and conspicuous edema flooding the lung parenchyma, and varying degrees of inflammatory changes (ICMR Report 1986). There are only a few experimental studies of a single inhalational exposure to MIC causing significant pulmonary pathology (Nemery et al. 1985; Jeevaratnam and Sriramachari 1994). The follow-up clinical and epidemiological studies revealed the persisting morbidity largely confined to the respiratory system (Sriramachari 1993). In fact, late autopsies of Bhopal victims showed that with the progress of time, the early exudative changes were replaced by chronic inflammatory lesions of the lung leading to pulmonary fibrosis and even fibrosing alveolitis. These long-term changes observed in more seriously affected survivors who succumbed subsequently were indeed the manifestations of a single exposure to "high but survivable concentrations" in severe, moderate and mild zones of the Bhopal gas disaster. In this regard, there were hardly any comparable long-term studies with the exception of the limited study by Dinsdale et al. (1987) following a single exposure to MIC. To understand the evolution of such lesions, the present investigation was undertaken to study the pulmonary pathology in rats exposed to a single high concentration or dose but were able to survive long enough to manifest the chronic pathological changes. Since in the preceding paper not only MIC but its hydrolytic derivatives, methylamine (MA) or *N,N'*-dimethylurea (DMU) were also found to produce pulmonary damage, their possible long-term effects too were investigated.

Materials and methods

Methyl isocyanate (99% purity) was synthesized and characterised in the Defence R&D Establishment, Gwalior as described earlier (Jeevarathinam et al. 1988). MA (40% aqueous solution) was obtained from E. Merck (India) and DMU from Sigma (USA). The details regarding inhalation exposure, subcutaneous injection and experimental set-up are as described in the previous paper (Jeevaratnam and Sriramachari 1994).

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For achieving the optimum dosage schedule high enough for a sufficient number of animals to survive upto the stipulated period, the investigation was carried out in three successive stages. Initially, a batch of 20 rats were exposed to 1 LC_{50} MIC; four rats were killed at the end of 1 week, while the remaining rats were allowed to live with the hope that at least some of them would survive for the subsequent time intervals. However, since none survived up to 4 weeks, the exposure concentration was scaled down to 0.75 LC_{50} and a fresh batch of 20 rats were exposed; four out of the surviving rats were killed at the end of 4 weeks. Since none of the rest survived for 10 weeks, a third batch of 20 rats were exposed to further lowered concentration of MIC (0.5 LC_{50}). A group of four rats was killed at the end of 10 weeks. Since none of the remaining animals survived the stipulated period of 16 weeks, the experiment was treated as terminated at the end of 10 weeks. In a similar manner, three doses, viz., 0.9 LD_{50} (n = 20), 0.75 LD_{50} (n = 20) and 0.5 LD_{50} (n = 20) of MIC administered s.c. for the study of changes at the end of 1, 4, and 10 weeks, respectively, so that at least four rats survived for the duration of the experiment. In the corresponding control groups for each duration of study, four rats each were subjected to either the inhalation procedure without the test material or s.c. administration of the vehicle, olive oil. Likewise for MA by the inhalation route for each period of study, four rats were exposed to 19 µmol/l (~1.0 LC_{50} MIC in terms of mol). In addition, either MA or DMU at 5.75 mmol/kg was injected s.c. into a group of four rats each for each period of the study.

Only a group of four rats of those that survived were killed at the end of each duration of the experiment by cervical dislocation. They were subjected to post-mortem examination. However, the animals that died during the course of the experiment were discarded to eliminate autolysed specimens. Although all the viscera were removed at autopsy and fixed in 10% neutral formalin, only the histopathological changes of the lungs are dealt in this communication. The tissues were processed through paraffin and stained by H&E, PAS, reticulin (silver) and collagen (trichrome) using standard procedures.

Results

The appearance and behavior of animals exposed to MIC and its hydrolytic derivatives have already been described in the preceding paper (Jeevaratnam and Sriramachari 1994). Nearly similar findings were found in all the rats after the exposure to varied concentrations of MIC. The animals continued to be moribund and scarcely consuming any feed or water. After 2 or 3 days, the general condition of the animals improved and they were moving about. Although many of them exhibited the signs and symptoms of acute respiratory distress syndrome (ARDS) up to 4 weeks, there was a gradual improvement in ARDS beyond 4 weeks. Thereafter, the animals were apparently normal without any signs of underlying gross pathology of the lung.

Gross findings

The gross pathological findings showed variegated lesions in the different groups with the progress of time. Maximal changes were seen in the MIC treated groups followed by MA and then by DMU. At the end of 1 week, the lungs of MIC treated animals were swollen, edematous and hemorrhagic. The hemorrhagic and patchy consolidation suggestive of grey hepatization was seen consistently in the groups of rats administered MIC s.c. At the end of 10 weeks, the lungs of both the groups showed features of consolidation.

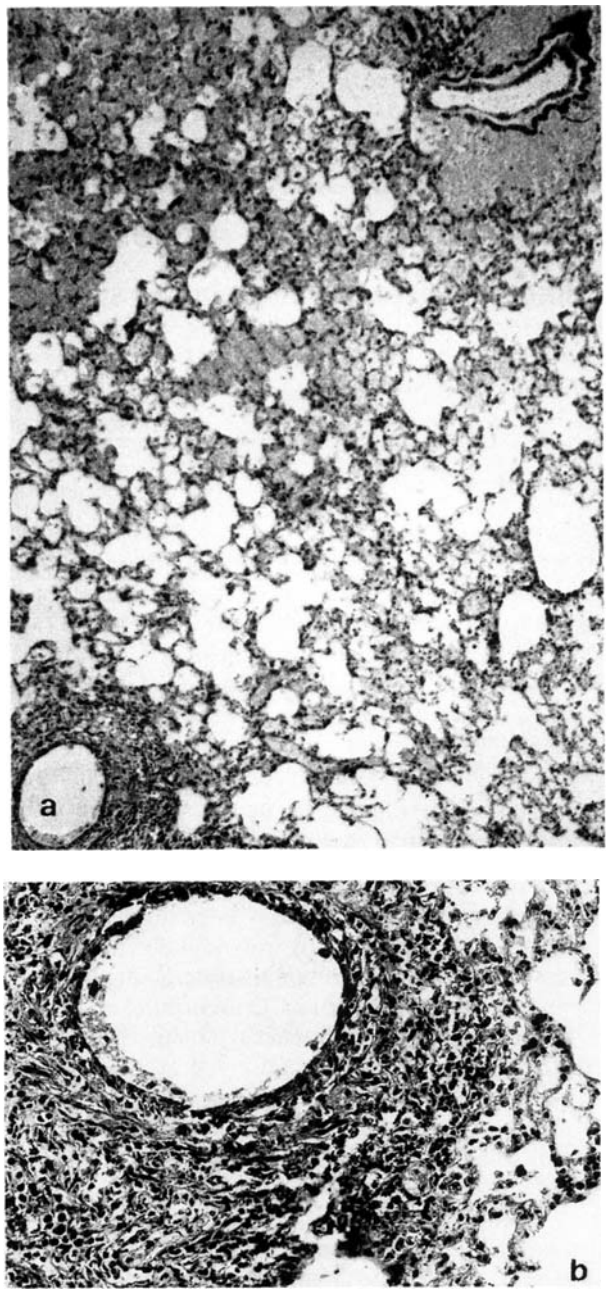


Fig. 1 a, b Lung of rat exposed to MIC (1 LC_{50}) after 1 week. **a** Shows eosinophilic necrosis of bronchiolar epithelium; peribronchial, perivascular, intra-alveolar and interstitial edema with varying degrees of cellular infiltration, H&E × 20, original. **b** Shows peribronchial, and interstitial infiltration by eosinophils, mononuclears, macrophages and fibroblasts extending into the inter-alveolar septa. H&E × 66, original

By contrast, both the MA treated groups showed a significantly persistent accumulation of hemorrhagic fluid in pleural cavity, often accompanied by a clot up to 4 weeks. In rats exposed to MA vapors through inhalation at the end of 1 week, the lungs were pale in color and translucent. However, at subsequent periods, there were no conspicuous changes except for reduction in the pleural effusion. In DMU treated rats, there was a mild consolidation of the lungs only up to 1 week while no apparent changes were observed at subsequent periods.

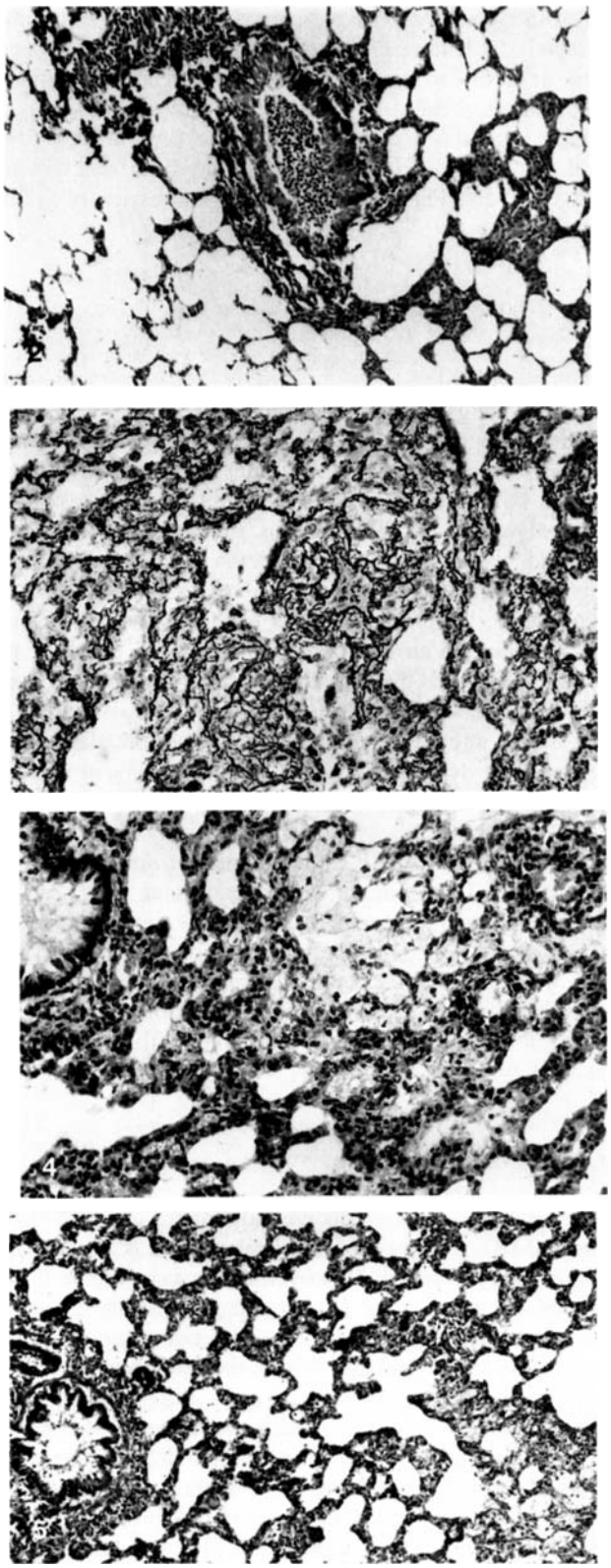


Fig. 2 Lung of rat exposed to MIC (0.75 LC50) after 4 weeks shows normal epithelial lining of bronchiole, the peri-bronchial inflammatory lesions spreading into the intra-alveolar septa with large foci of interstitial pneumonitis. H&E × 50, original

Fig. 3 Lung of rat exposed to MIC (0.75 LC_{50}) after 4 weeks shows abundant argentophilic fibers in the thickened interstitial septa. Silver × 100, original

Fig. 4 Lung of rat exposed to MIC (0.5 LC_{50}) after 10 weeks shows normal bronchial lining, interstitial edema with intense pneumonitis, intra-septal and intra-alveolar foamy macrophages. H&E × 66, original

Fig. 5 Lung of rat exposed to MIC (0.5 LC_{50}) after 10 weeks shows peribronchial and interstitial fibrosis with prominent desquamative changes. Trichrome × 50, original

Histopathological observations

At each period of study, comparatively more severe lesions were observed in the MIC treated groups followed by MA groups while DMU treated animals showed mild changes.

MIC by inhalation

One week

The "striking feature of acute eosinophilic necrosis of the bronchial and bronchiolar epithelium" seen after 24 h, except for tiny foci virtually disappeared in the group of animals killed 1 week after exposure to MIC vapors. The lumen of the bronchioles were practically patent throughout. There was no evidence of plugging of bronchioles by either shed epithelial debris or by inflammatory exudates. Extensive patches of intra-alveolar edema of the acute phase were no longer present. Nevertheless, there was marked diffuse interstitial edema with indistinct inter-alveolar septa. There was also perivascular and peribronchial edema and varying degrees of interstitial pneumonitis (Fig. 1). The cells were predominantly mononuclears, histiocytes and a few indistinct fibroblasts. In spite of heavy cellular infiltrates, the absence of argentophilic reticulin fibers in the silver preparations indicated inconspicuous fibroblastic proliferation.

Four weeks

Section of the lungs showed apparently normally regenerated bronchial epithelium. It is noteworthy that the bronchial epithelium showed occasionally a relative increase of goblet cells which are intensely PAS positive, indicative of minor variations in the process of epithelial regeneration. The bronchial epithelial lining was apparently without heaping of the cells or crowding of the lumen, except for an occasional collection of inflammatory cells. However, there was no evidence of bronchiolitis obliterans in any of the areas examined. There was severe interstitial pneumonitis with obliteration of alveolar outlines, and varying degrees of inflammatory reaction in the thickened septa (Fig. 2). In places, the alveoli were seen filled with desquamated foamy macrophages and/or edematous fluid. The widened inter-alveolar spaces were five to eight cells thick and occasionally an even larger island of interstitial cells was observed, crowding out the alveoli and resulting in areas of atalectesis and emphysematous entrapped air. In

a few places, such alveoli were markedly distended giving a characteristic feature of emphysema occupying the space of three or four or even more alveoli. The large foamy macrophages were seen not only filling the alveoli but also the interstitial compartment. The presence of fibroblasts with marked increase in argentophilic reticulin and collagen fibers (Fig. 3) indicated the progression of pulmonary fibrosis.

Ten weeks

Organization of the above histological changes was apparent in the lungs of rats exposed to 0.5 LC_{50} MIC. The process of restoration of bronchial epithelium was practically complete and even the goblet cells were not seen. There is no evidence of any inflammatory changes in the bronchial wall itself. The lumens were patent. There was little evidence of inflammatory edema in the peribronchial, interstitial tissue spaces and alveoli. The continuity of the alveoli to the lower airways was further disrupted, leaving foci of entrapped air and markedly distended emphysematous areas. At this period, the outstanding features were organized interstitial pneumonitis with extensive areas of proliferated interstitial cells, notably mononuclear cells, foamy histiocytes and fibroblasts, and resulting in disorganization of lobular architecture. The histological feature of the cellular infiltrates revealed areas of intra-alveolar desquamative foamy cells and presence of similar cells in the highly cellular interstitial compartment (Fig. 4). In addition, in the interstitium there was a marked increase in fibroblasts and number of argentophilic fibers and frank areas of collagenous strands as seen in the trichrome preparations. These features clearly resemble the description of a diffuse interstitial pulmonary fibrosis (DIPF) (Fig. 5).

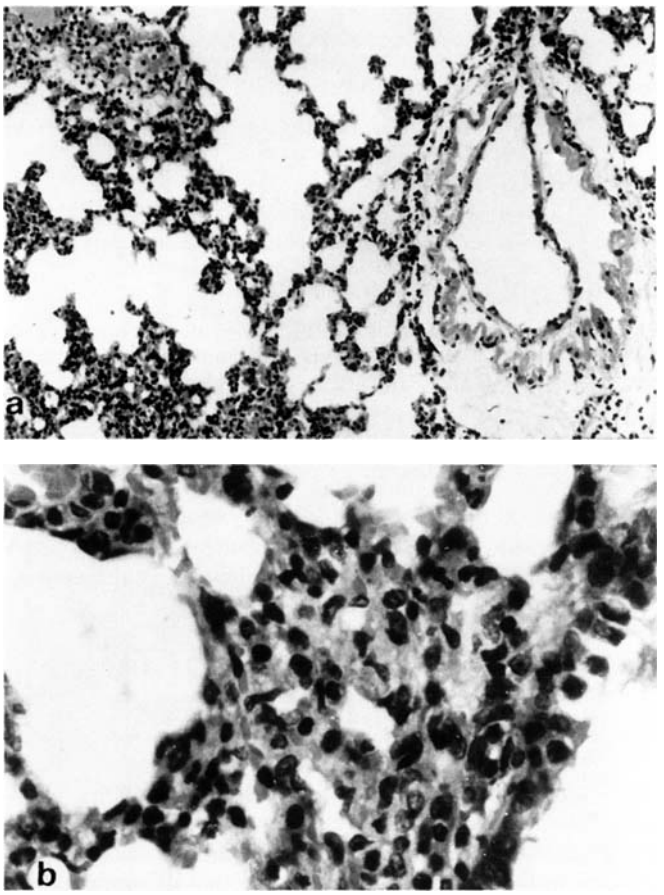
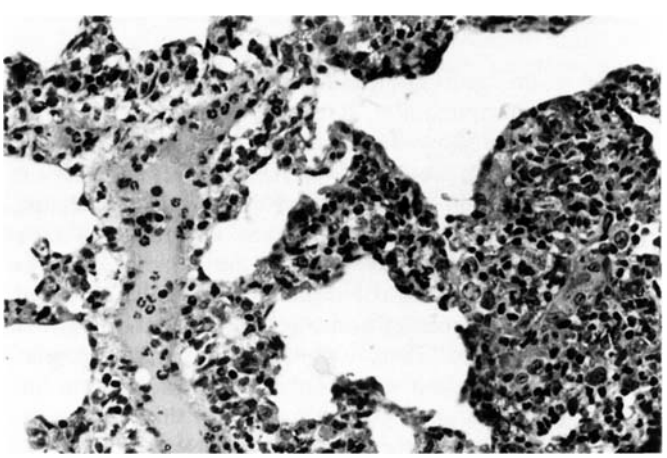


Fig. 6 a, b Lung of rat administered MIC (0.9 LD_{50}) s.c. after 1 week. **a** Shows marked congestion with interstitial pneumonitis, vasculitis, perivascular edema with separation of muscular coats of blood vessel, H&E × 50, original. **b** Shows marked thickening of alveolar septa, congestion with interstitial pneumonitis containing inflammatory cells, mononuclears, macrophages and fibroblasts. H&E × 200, original

MIC by subcutaneous route

In the animals subjected to s.c. administration of a single dose of MIC, a more or less similar trend of progressive changes was observed. However, there were minor differences in the severity of changes and the relative preponderance of cellular elements at the different periods studied. Hence, only the significant points are described. As in the acute phase, at all the periods of study, the bronchial lining continued to be normal. There were neither plugs of inflammatory cells nor shed epithelial debris. The bronchial walls also did not show any evidence of inflammatory changes. The edematous changes in peribronchial, intra-alveolar and interstitial tissue were also less pronounced. The outstanding feature was the very prominent cellular reaction, than in the inhalation group. By comparison, the widened inter-alveolar spaces contained anywhere between 8 and 15 cells. The cells were progressively transformed from mononuclears and macrophages to fibroblasts. These changes were confined predominantly to the interstitial compartment and not within the alveoli.

Fig. 7 Lung of rat administered MIC (0.75 LD_{50}) s.c. after 4 weeks shows marked hyperemia, cellular interstitial pneumonitis and vascular endothelial damage. H&E × 100, original



One week

Although there was no distinct intra-alveolar edema, there were fairly severe interstitial edema extending into the perivascular spaces and even splitting the adventitial and medial muscular coats (Fig. 6). Lungs were also markedly congested with pronounced interstitial pneumonitis.

Four weeks

Sections of the lungs showed marked hyperemia and cellular interstitial pneumonitis without any recognizable edema (Fig. 7). Silver staining showed sparse argentophilic fibers in the widened otherwise cellular septa indicating early stages of fibrosis.

Ten weeks

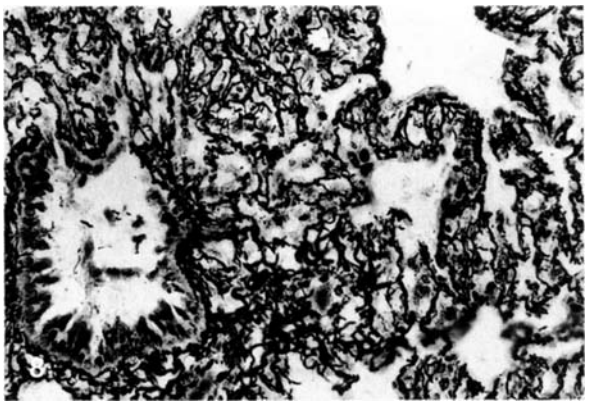
Lung parenchyma showed extensive organized interstitial pneumonitis with disorganization of alveolar pattern. Multinucleated giant cells were also seen in several areas. Reticulin staining showed extensive peribronchial, perivascular and interstitial fibrosis with abundance of argentophilic fibers (Fig. 8). The trichrome staining of the corresponding area showed marked fibrosis with diffuse collagenous interstitial pneumonitis.

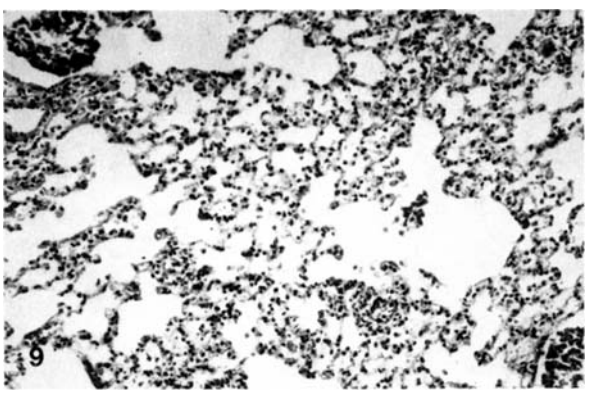
Methylamine

At all periods of the study, the bronchial lumen and the epithelial lining were apparently normal in both the MA groups.

One week

A peculiar and extensive edema was observed at the end of 1 week in rats exposed to MA vapors through inhalation route. The lungs were uniformly pale and had a washed out appearance. Histologically, lung parenchyma was filled with transparent clear aqueous fluid with the widespread separation of tissue spaces. Thus, the mucosal lining was lifted off the submucosa (Fig. 9). Similarly, the perivascular areas were filled with clear edematous fluid extending throughout the pulmonary interstitium and alveoli. This phenomenon was seen to extend into the walls of blood vessels whose adventitial muscular coats were widely separated (Fig. 10). The faintly PAS positive reaction indicated that the fluid is an exudate rather than a transudate. The above phenomenon was not observed at any other period, including the acute phase. In both the MA groups, the interstitial spaces showed varying degrees of infiltrates characterized by mononuclear cells and histiocytes without any foamy cells. The connective tissue fibers were sparse and inconspicuous as revealed by both the silver and trichrome preparations.





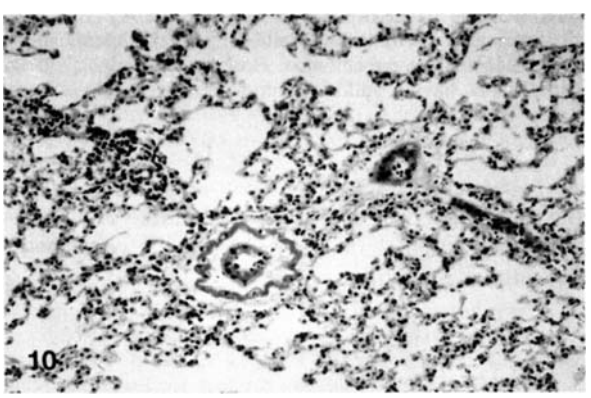


Fig. 8 Lung of rat administered MIC (0.75 LD_{50}) s.c. after 4 weeks shows extensive peribronchial, perivascular and interstitial fibrosis with abundance of argentophilic fibers. Silver × 100, original

Fig. 9 Lung of rat exposed to MA (19 µmol/l) after 1 week shows separation and folding of the bronchial epithelium, dilatation of alveolar ducts and interstitial pneumonitis. H&E × 50, original

Fig. 10 Lung of rat exposed to MA (19 µmol/l) after 1 week shows marked widening of vascular coats by edematous fluid and moderately severe interstitial pneumonitis. H&E × 50, original

Four weeks

A moderate to severe interstitial pneumonitis without edema was noticed in lungs of rats exposed to MA by either route (Fig. 11a), the severity being more pronounced

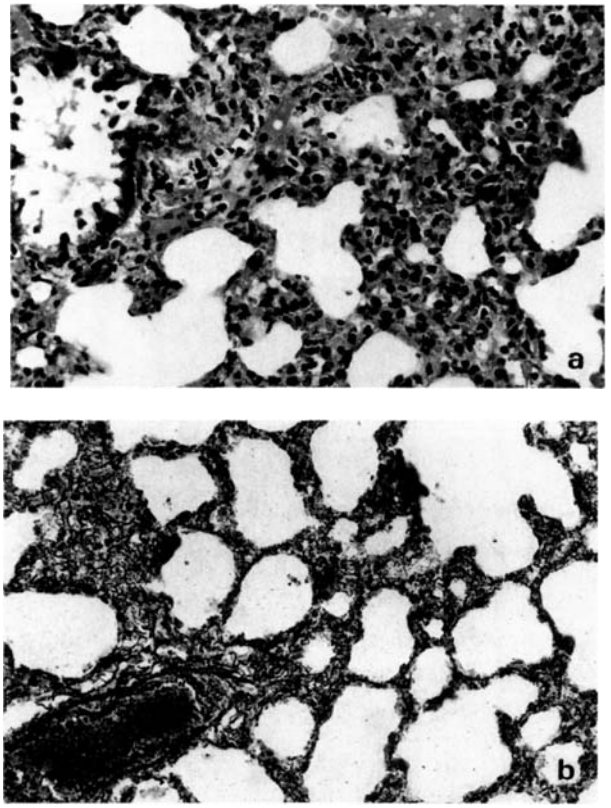


Fig. 11a, b Lung of rat exposed to MA (19 μmol/l) after 4 weeks. **a** Shows normal epithelial lining, mild interstitial edema and moderately severe interstitial pneumonitis, H&E × 100 original. **b** Shows interstitial septa having mild argentophilic fibers. Silver × 100, original

in the subcutaneous route of exposure. The reticulin staining showed moderately severe fibrillogenesis in the highly cellular interstitial septa (Fig. 11 b).

Ten weeks

In both the groups, the lung showed severe interstitial pneumonitis extending into peribronchial and perivascular areas (Fig. 12 a). The silver preparation showed abundance of collagen fibers in the corresponding areas (Fig. 12 b). By comparison, the intensity of the above lesions is less in MA treated rats than in the MIC treated rats.

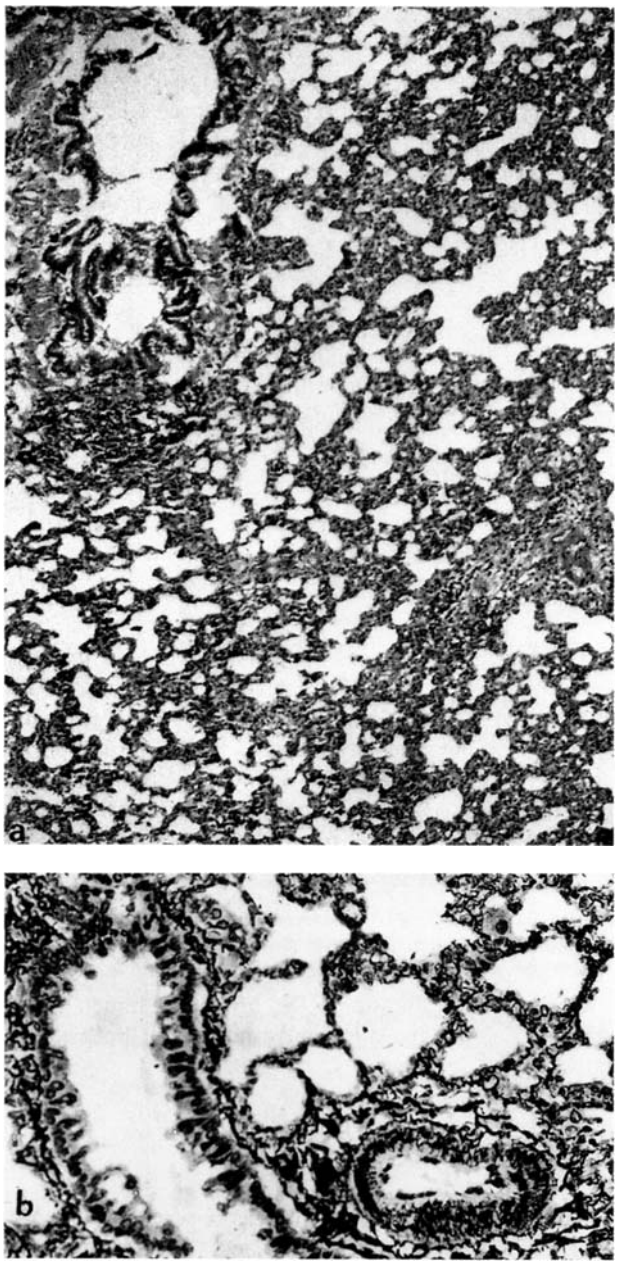


Fig. 12a, b Lung of rat exposed to MA (19 μmol/l) after 10 weeks. **a** Shows irregular and partially separated bronchial epithelium without any inflammatory reaction in the lumen or wall; peribronchial lymphocytic infiltration; foci of vasculitis and surrounding areas of interstitial pneumonitis extending into the inter-alveolar septa. H&E × 33, original. **b** Shows abundant argentophilic fibers in the alveolar septa and peribronchial regions. Silver × 100, original

N,N′-Dimethyl urea

In the DMU treated animals, at none of the time intervals were there any noteworthy changes in the bronchi and bronchioles. There was also no evidence of edema at any period. However, at the end of 1 week there was fairly prominent interstitial pneumonitis with widening of inter-alveolar septa and a moderate cellular infiltrates of mononuclear cells. Neither at this period nor later was there any evidence of transformation to macrophages, fibroblasts, argentophilic reticulin fiber formation and much less presence of collagen. In fact, from 4 weeks onwards, there was conspicuous decrease in the interstitial pneumonitis. At the end of 10 weeks the lungs are practically normal.

Discussion

Pulmonary edema was one of the outstanding manifestations in the lungs of early autopsies of Bhopal gas victims. The same has been amply confirmed in the present study following a single exposure to a high concentration of MIC, not only at the end of 24 hours but even up to 4 weeks. The lesions are more than comparable with those described by Nemery et al. (1985).

The foregoing results clearly demonstrated that as with acute exposure to MIC, a single exposure to near or sublethal concentration/dose of MIC caused long-term permanent damage of the lung characterized by interstitial pneumonitis and progressive fibrosis. Between the two routes of exposure to MIC, only in the inhalation group were the alarming lesions of bronchial necrosis and pulmonary edema both intra-alveolar and interstitial prominent. On the other hand, the inflammatory response was more pronounced with the s.c. route. With the progress of time, apart from minor variations, the evolution of lesions appeared to be similar, culminating in the development of significant interstitial pneumonitis and fibrosis. From the available literature, it would appear that the present changes are far more marked. Both in terms of the intensity of interstitial pneumonitis and laying down reticulin and collagen fibers they are more severe than those observed by Nemery et al. (1985) and Dinsdale et al. (1987). The only notable difference even at the comparable period of 3–4 weeks was their observation of bronchiolitis obliterans. A careful search did not reveal such lesions in the present study. There is no ready explanation for this discrepancy even though the corresponding effective dosage of exposure is certainly higher than that of Dinsdale et al. (1987). The marked fibrosis of the lung seen at 4 weeks and progression to more severe lesions at the end of 10 weeks, is perhaps of greater significance, particularly in the context of the occurrence of pulmonary fibrosis in the late autopsies in Bhopal gas victims (Sriramachari 1993).

Irrespective of the route of administration of molar equivalence of MIC or MA, the response was more pronounced in the MIC groups. This suggests that perhaps, MIC per se is relatively more deleterious. However, an interesting feature of the present study is the ability of MA vapors (inhalation) to cause pulmonary edema at 1 week, although not at the other periods. On the other hand, the associated interstitial pneumonitis progressing to fibrosis is noteworthy. Certainly, MA, a known hydrolytic derivative of MIC generally believed to be innocuous, also seems to contribute to the long-term pulmonary damage. However, it is not certain to what extent the long-term effects of MIC are attributable to its in vivo transformation to MA. Although it is reported that a single exposure to a near-lethal concentration of MA results in pulmonary damage (Beard and Noe 1981), the present findings reveal that even exposure to a sublethal concentration/dose by either inhalation or s.c. producing significant long-term pulmonary damage is of importance. These results suggest that not only the reported findings of large quantity of MA in the tank residue and aerosol (Union Carbide Report 1985; Varadarajan et al. 1985; Rao et al. 1991) but also the probable in vivo transformation of MIC to MA might have partly contributed to the cumulative effect in the pathogenesis of pulmonary fibrosis in the Bhopal gas victims. Though the mechanisms by which either MIC or MA elicit the observed long-term effects are not known presently, the fact that the lung is the target organ for both MIC and MA, which cause the progressing changes leading to a unified picture of chronic pulmonary fibrosis, merits further consideration.

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References

Beard RR, Noe JT (1981) Aliphatic and alicyclic amines. In: Clayton GD, Clayton FE (eds) Patty's industrial hygiene and toxicology, 3rd edn. Wiley, New York, pp 3135–3173

Dinsdale D, Nemery B, Sparrow S (1987) Ultrastructural changes in the respiratory tract of rats following methyl isocyanate inhalation. Arch Toxicol 59: 385–390

ICMR Report (1986) Health effects of the Bhopal gas tragedy. Indian Council of Medical Research, New Delhi

Jeevaratnam K, Sriramachari S (1994) Comparative toxicity of methyl isocyanate and its hydrolytic derivatives in rats. I Pulmonary histopathology in the acute phase. Arch Toxicol

Jeevarathinam K, Selvamurthy W, Ray US, Mukhopadhyay S, Thakur L (1988) Acute toxicity of methyl isocyanate administered subcutaneously in rabbits: changes in physiological, clinicochemical and histological parameters. Toxicology 51: 223–240

Nemery B, Dinsdale D, Sparrow S, Ray DE (1985) Effects of methyl isocyanate on the respiratory tract of rats. Br J Ind Med 45: 799–805

Rao GJ, Saraf AK, Purkait R, Sharma VK, Jadhav RK, Heeresh Chandra and Sriramachari S (1991) J Ind Acad Forens Sci 30: 13–18

Sriramachari S (1993) Observations related to health effects of Bhopal toxic gas leak. Proceedings of International Toxicovigilance Conference, Cardiff, UK, April 1–3, 1993 (in press)

UCC Report (1985) Bhopal methyl isocyanate incident investigation team report. Union Carbide Corporation, Danbury, Connecticut. March

Varadarajan Committee Report on scientific studies on the factors related to Bhopal toxic gas leakage, December, 1985