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Pulmonary response to methylcyclopentadienyl manganese tricarbonyl treatment in rats: injury and repair evaluation

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Summary. Methylcyclopentadienyl manganese tricarbonyl (MMT), an organometallic compound, used as an antiknock additive in fuels, may produce alveolar inflammation and bronchiolar cell injury.

The aim of the experimental study on female rats was to determine by morphological examination and sensitive biomarkers, the course of the injury and repair process following a single i.p. injection of 5 mg/kg MMT. The animals were sacrificed 12, 24, 48 hours or 7 days post-exposure (PE). The first biochemical changes 12 h PE showed an increase in GSH-S-transferase (GST) activity in the lung parallel to the earliest observed morphological changes -vacuolation and swollen cytoplasm in type I pneumocytes. Alterations in type I pneumocytes were most prevalent in rat lung 24 h PE. Clara cells with dilated smooth endoplasmic reticulum membranes and cytoplasmic vacuolation could be observed. Compared to the values found for controls, Clara cell protein (CC16) in the bronchoalveolar lavage fluid (BALF) at 24 and 48 h PE decreased by 58% and 55%, respectively. At the same time (at 24 and 48 h), the total protein concentration in BALF increased 5 and 7 times, respectively. A significant rise in hyaluronic acid (HA) level was observed 24 and 48 h PE. Divided type II pneumocyte cells and Clara cells in their mitotic phase were observed in immunocytochemistry (detecting BrdU binding into DNA) 48 h PE. Seven days after MMT administration, fibroblasts, macrophages, collagen and elastin fibres could be seen in the alveolar walls as well as neutrophils, lymphocytes, and alveoli macrophages in the alveolar lumen. We conclude that injury and repair of bronchial epithelium cells, especially of Clara cells and type II pneumocyte cells, play an important part in MMT toxicity, probably depending on the antioxidant status of these cells. The sensitive biomarkers of CC16 and hyaluronic acid in BALF and serum reflect lung injury and indicate the time course of pulmonary damage and repair processes.

Key words: MMT, Lung, Clara cell protein, Hyaluronic acid, BrdU

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Introduction

A number of environmental agents and commonly used chemical compounds show pneumotoxicity and can cause pulmonary disorders. The toxicity of methylcyclopentadienyl manganese tricarbonyl (MMT), an organometallic compound used as an antiknock additive in a variety of fuels, was investigated in animal experiments on several species (Hinderer, 1979; Hanzlik et al., 1980; Hakkinen and Haschek, 1982; Penney et al., 1985; McGinley et al., 1987). It was concluded that this compound produced severe alveolar inflammation with only mild bronchiolar airway cell injury. The rat was found to be 26 times more sensitive to MMT than the mouse, and 45 times more sensitive than the hamster, as judged by the comparative LD50 values after i.p. administration (Hakkinen and Haschek, 1982). Bronchiolar damage was most severe in the mouse and only mild in rats in which interstitial inflammation and oedema dominated. Degenerative changes as shown by cytoplasmic vacuolation were found in rat Clara cells. Activation by organomanganese (MMT) compounds in Clara cells probably leads to the production of active metabolites that are capable of reaching targets in rat alveolar type II cells (Clay and Morris, 1989; Blanchard et al., 1996). It is known that many metabolically activated pneumotoxic compounds, such as furans

(Boyd and Reznik-Schuller, 1984), naphthalene (Lin et al., 2005), 3-methylindole (Woods et al., 1993) or bromobenzene (Forkert, 1985), induce Clara cell injury. Moreover, it was shown that Clara cells are involved in the renewal of distal airway epithelium after the injury (Hakkinen and Haschek, 1982). Many studies in a number of species have indicated the Clara cell as a possible progenitor cell for bronchiolar epithelium in the repair process (Hackett et al., 1992, Van Winkle et al., 1995).

In the alveolar epithelium, type II pneumocytes play a significant role in the rebuilding processes after lung injury since they are the progenitor cells for both type I and type II epithelial cells (Durham and Gijbels, 1989; Sulkowska et al., 1996). Type I pneumocytes cover approximately 90-95% of the alveolar surface; the remaining part being type II pneumocytes where the synthesis and secretion of surfactant occur. An interaction between type II pneumocytes and fibroblasts also plays a significant role in the fibrosis processes.

Clara cell 16 kDa protein (CC16), a counterpart of rabbit uteroglobin (Miele et al., 1987; Mantile et al., 1993) is a potent inhibitor of phospholipase A2 and is thought to have some role in the anti-inflammatory activity (Lesur et al., 1995). This protein can be detected by a sensitive immunoassay in bronchoalveolar lavage fluid (Bernard et al., 1992). The BALF procedure allows cells and soluble components to be collected from the distal part of the respiratory tract. Some of these components such as fibronectin, hyaluronan and collagen can serve as biomarkers, since their elevated level in BALF indicates interstitial lung disease (Rennard and Crystal, 1982; Hallgren et al., 1985; Bjermer et al., 1987; Blaschke et al., 1990). They are also involved in the pathogenic mechanisms leading to fibrosis (Davidson, 1990).

The aim of this study was to determine, using morphological and biochemical markers, the time course of injury and repair processes in the rat lung after treatment with a pneumotoxic agent. Special attention was paid to the relationships between the morphology of Clara cells and type II pneumocytes, and the levels of Clara cell protein in BALF and serum during the injury and repair processes.

Material and methods

Animals and treatment

Female rats of the Wistar strain were used in the experiment. All animals were kept in propylene plastic cages on hardwood bedding at 22-25°C, a relative humidity of 50%, on a 12-hour light-dark cycle, and fed with laboratory chow Murigran and tap water ad libitum. Animals were divided into two groups: a group of 40 rats were injected i.p. with methylcyclopentadienyl manganese tricarbonyl (MMT, Aldrich) in a single dose of 5 mg/kg b.w. dissolved in corn oil (1 ml/kg); 40 control rats were given corn oil (1 ml/kg) by i.p. injection. The tests were performed 12 h, 24 h, 48 h or 7 days after injection. The animals were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.) and exsanguinated via the abdominal aorta. Blood samples were stored at 4°C for 3 hours and then centrifuged at 2000 g for 10 minutes and the serum samples were stored at -20°C until further analysis. The trachea was cannulated when respiration had ceased and bronchoalveolar lavage was performed. The lungs were lavaged with normal saline, using a total volume of 10 ml. The bronchoalveolar lavage fluid was centrifuged (200 g, 10 min, 4°C) and the cell-free supernatant was used for testing. For pathomorphological examination the lungs were quickly removed. The lungs of animals from each group were fixed by intratracheal infusion (20 cm of hydrostatic pressure) with glutaraldehyde/paraformaldehyde (1%/0.5%) in cacodylate buffer (pH7.4) and processed for light- and electron-microscopy.

Biochemical analysis

In BAL fluid the following measurements were performed: Clara cell protein (CC16), total protein and hyaluronic acid (HA). Clara cell protein concentration was determined by latex immunoassay (Halatek et al., 1998). Specific rabbit antibodies against CC16 and a standard for CC16 based on the purified protein were obtained as described earlier (Halatek et al., 1998). CC16 was determined in BALF without sample pre-treatment. To eliminate the possible interference (complement, chylomicrons), the serum samples were pre-treated by heating at 56°C for 30 min. and by the addition of polyethylene glycol 600 (16%, v/v 1/1) and trichloroacetic acid (10%, v/v 1/40). After overnight sedimentation, the samples were centrifuged and CC16 was determined in supernatants. Total protein concentration in BALF was determined. Hyaluronic acid (HA) was measured in BALF by radiometric assay according to Engstrom-Laurent et al., (1985), using Pharmacia HA kit (Uppsala, Sweden).

GSH-S-transferase (EC 2.5.1.18) in post-mitochondrial supernatants of rat lung was determined with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974).

Immunocytochemistry

At each time-point the animals were injected i.p. with BrdU (100 mg/kg) in phosphate-buffered saline (PBS) 1 h before anaesthesia. Lung tissue sections were then embedded in paraffin and sectioned. After standard dewaxing and rehydration, lung sections were stained with monoclonal anti-BrdU antibody and NBT-x phosphatase kit (Boehringer-Mannheim) to detect BrdU incorporated into DNA of proliferating cells. To eliminate nonspecific binding of the primary antibody, the sections were blocked with 1% bovine serum albumin after a 15 min digestion with 2N HCl (Van Winkle et al., 1995). The avidin-biotin peroxidase

procedure was used to identify antibody binding sites.

Light and transmission electron microscopy

After fixation with glutaraldehyde/paraformaldehyde, lung sections were postfixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in epoxy resin (Poly-Bed 812). Semithin sections (1μm) from lung were cut on LKB Ultratome III with glass knives and stained with toluidine blue. The sections were examined and some areas were selected for electron microscopy evaluation. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined using JEM 100-C (JEOL) electron microscope at 80 kV.

Statistical analysis

All statistical analyses were performed using the Sigma Stat software. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the multiple comparison test. The level of significance was set at $p<0.05$.

Results

Table 1 shows the concentrations of the study parameters: CC16, total protein, and HA in BALF, CC16 in serum and GST in lung as well as lung weight/100g b.w. 12 h, 24 h, 48 h and 7 days following a single i.p. injection of MMT in the study group and the respective

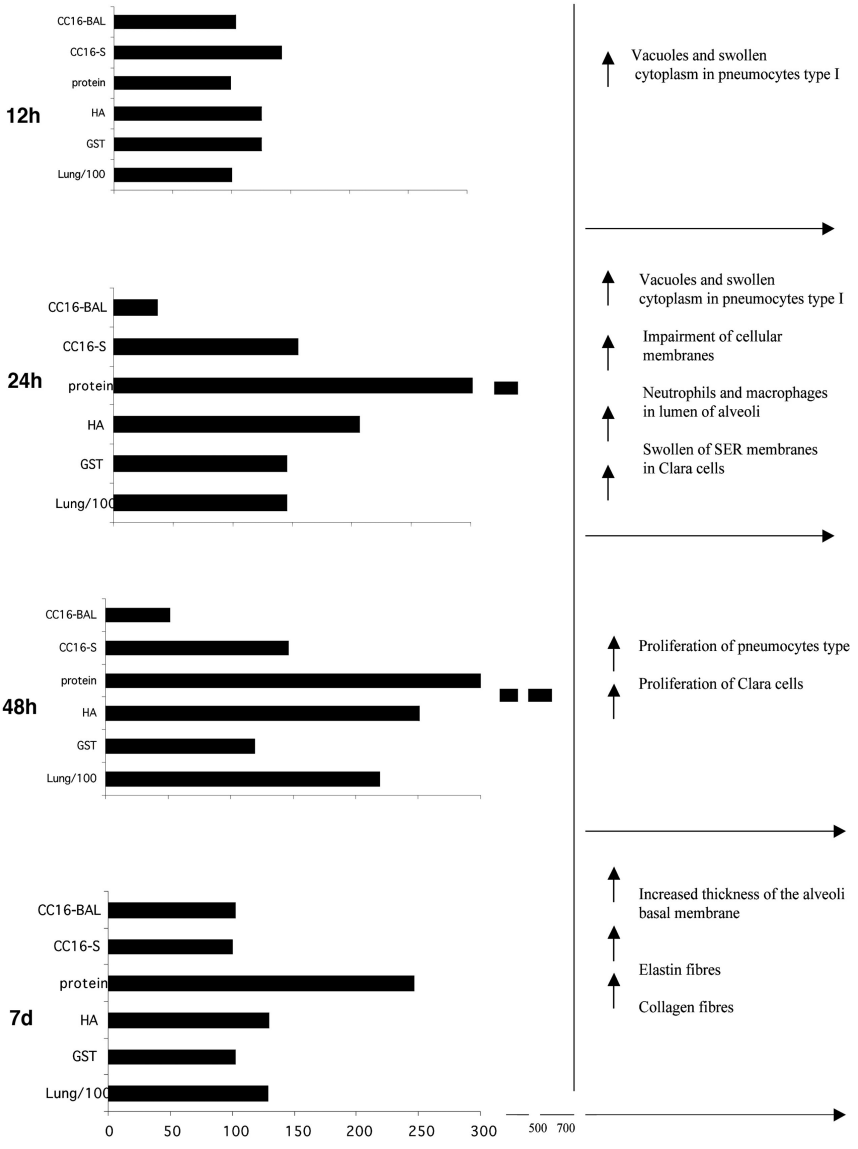

Fig. 1. Biochemical and morphological changes following MMT injection, per cent values for the exposed group vs. matching controls, at a given time-point.

values for the control group receiving pure corn oil injection. Figure 1 presents the per cent values of the study parameters in the exposed and control groups and displays the most important morphological findings post-exposure. The first statistically significant changes concerned GSH-S-transferase activity. 12 h post injection, an increase was observed in GST activity in the lung of rats intoxicated with MMT ($p<0.05$). A statistically significant increase in this activity was also found 24 h PE, but not after 48 h and 7 days PE (Table 1, Fig. 1).

Most of the study parameters presented statistically significant changes 24 h PE (CC16, total protein, HA, GST, lung/100g b.w.) and 48 h PE (total protein, HA, CC16-S, lung/100g b.w.). Seven days PE, this referred only to total protein and lung weight/100g b.w. (Table 1, Fig. 1).

The mean CC16 concentration in BALF of rats intoxicated with MMT decreased by 63 % at 24 h PE, significantly compared to controls, $p<0.05$ (Table 1, Fig. 1). Decreased concentrations of CC16 protein in BALF of rats treated with MMT were also found after 48 h significantly compared to controls, $p<0.05$. Seven days after exposure to MMT, CC16 concentration increased

Table 1. Concentration of CC16, total protein and HA in BALF, CC16-S in serum and GST activity in lung and weight of lung/100 g b.w. following MMT i.p. injection at 5 mg/kg b.w.

	Treatment group				Control **
Parameters/Time of examination – PE	12 h (N=5)	24 h (N=5)	48 h (N=5)	7 days (N=5)	(N=20)
Lung weight/100 g; b.w.	0.96±0.01	1.27±0.03 *	2.04±0.20 *	1.26±0.24 *	0.90±0.10
Total protein; mg L^{-1}	1252±422	6957±2651*	8548±3482 *	2961±687 *	1262±586
CC16-BALF; μg L^{-1}	5767±118	2500±180 *	2667±2146	6257±1723	5917±1472
CC16-S; μg L^{-1}	26.2±11.5	28.3±20.9	28.4±6.5 *	26.8±9.8	20.0±10.1
HA; μg L^{-1}	13.6±6.1	35.7±11.9 *	42.0±11.3 *	20.9±2.9	16.8±7.9
GST; nmol/CDNB-GSH/min/mg protein	257±12 *	276±20 *	235±18	226±26	206±21

* Values are mean ± SD significantly different from control at $p<0.05$; ** Control values pooled from different PE groups.

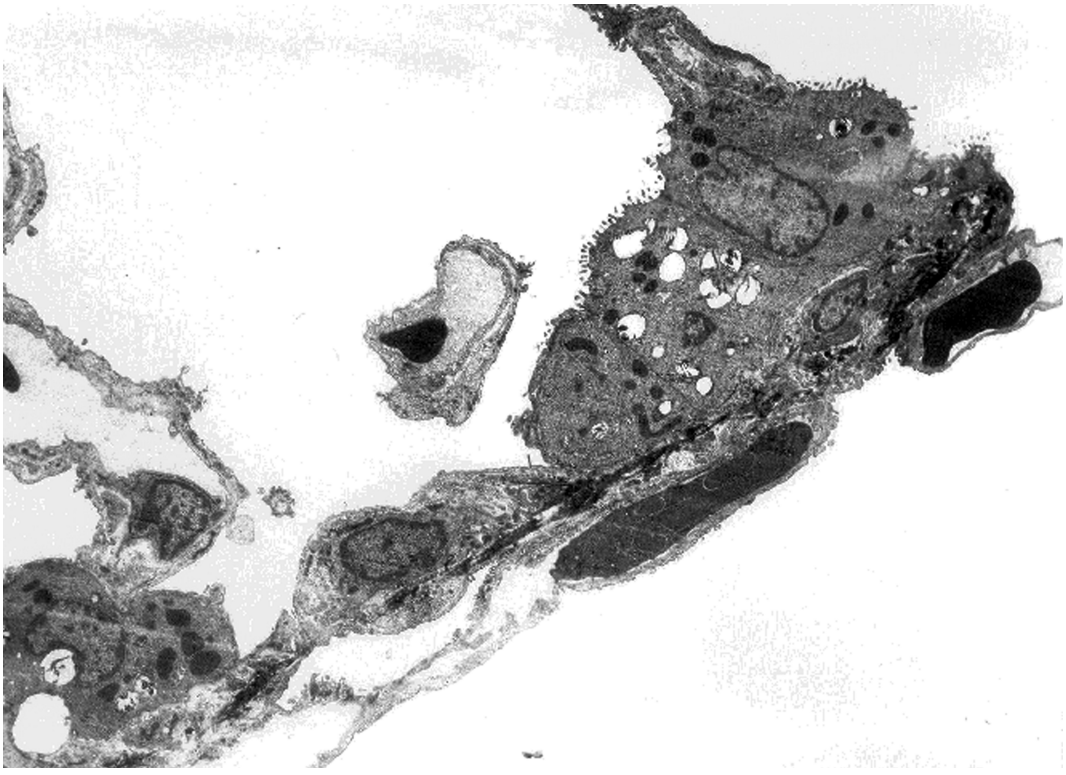

Fig. 2. Alveolar epithelium of control rat. Normal image of type I and type II pneumocyte and capillary. x 3200

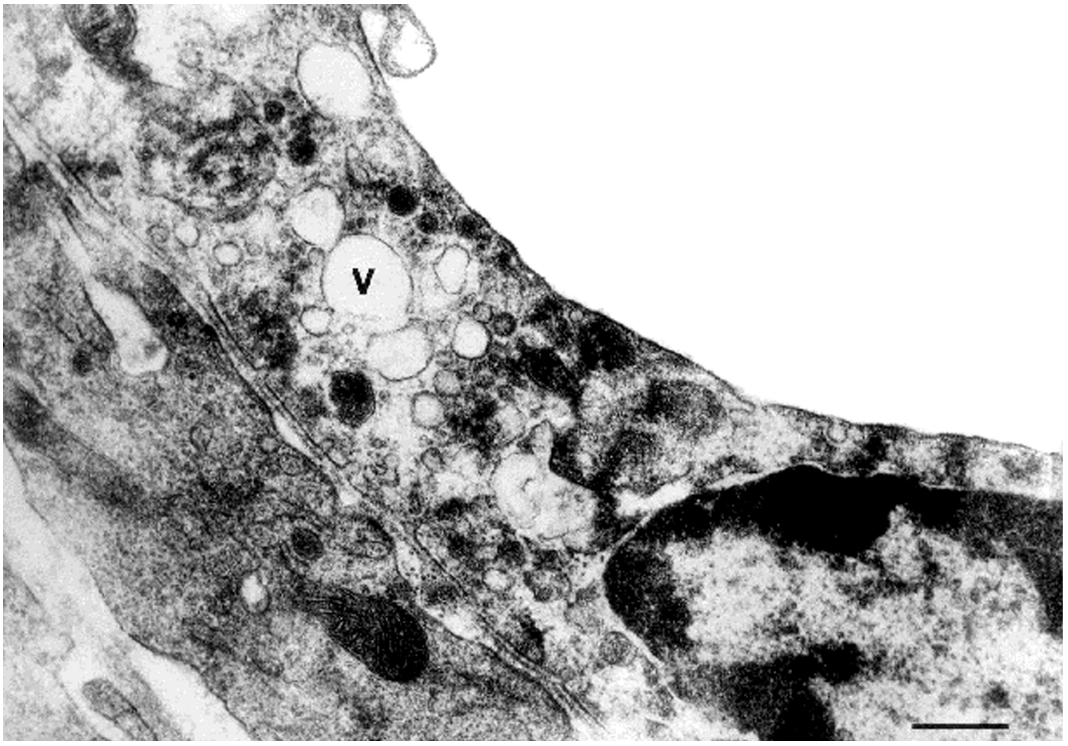

Fig. 3. Alveolar epithelium of rat 12 h after MMT treatment. Type I pneumocyte with vacuoles (V) and swollen cytoplasm. x 16000

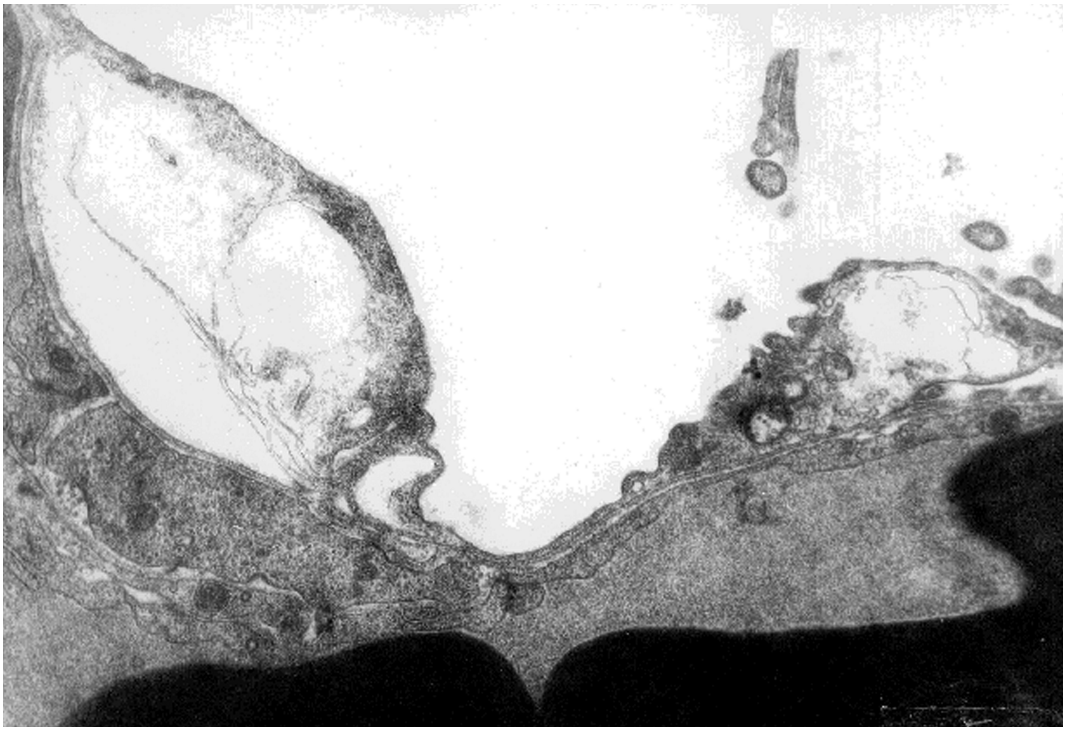

Fig. 4. Alveolar epithelium of rat 24 h after MMT injection. Type I pneumocyte with swollen cytoplasm, and disruption of cellular membranes. x 13000

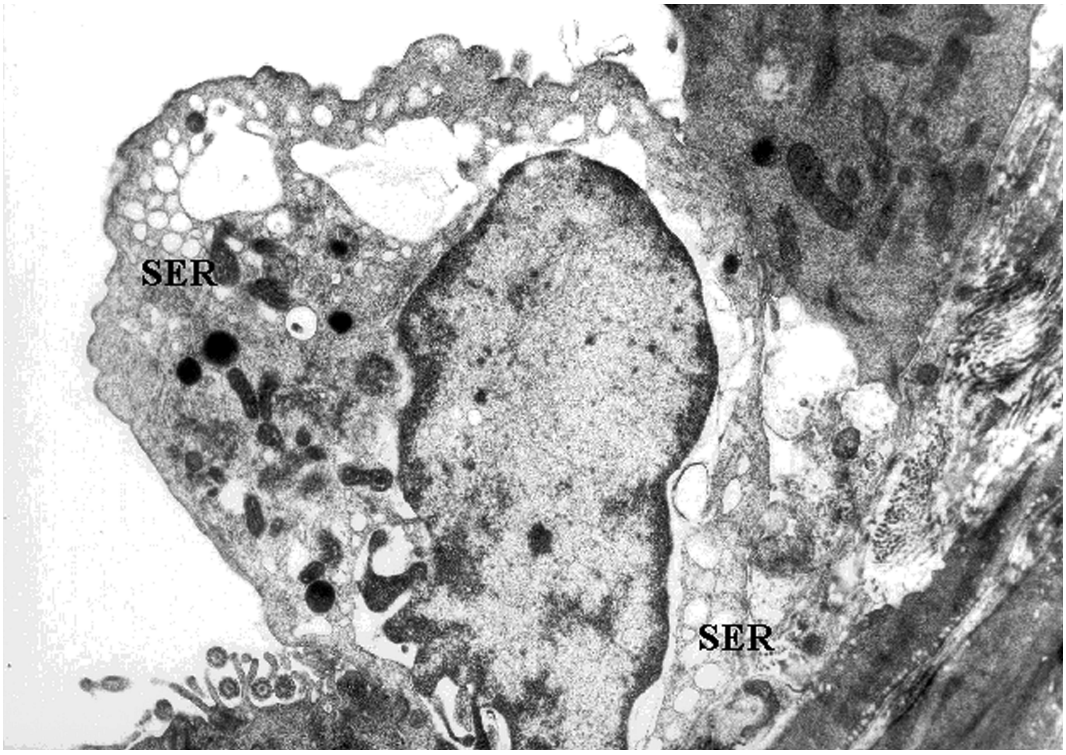

Fig. 5. Ultrastructure of bronchiolar epithelium of rat 24 h after MMT injection. Clara cell with dilated smooth endoplasmic-reticulum membranes (SER) and vacuolated cytoplasm. x 10000

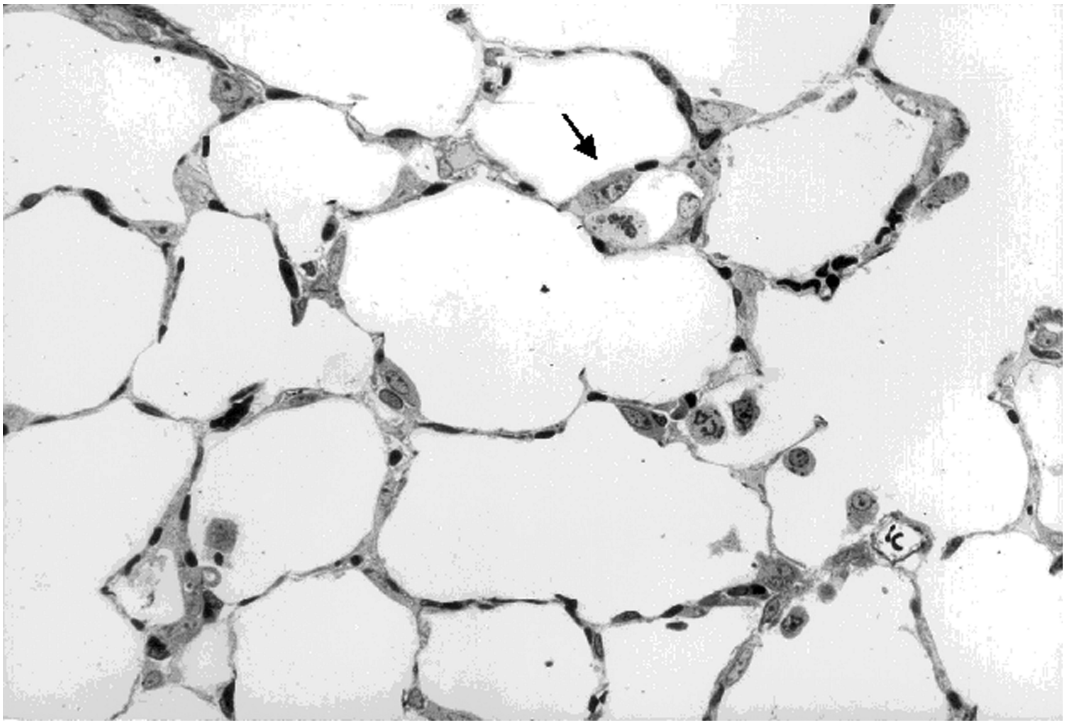

Fig. 6. Lung of rat 48 h after MMT treatment. Proliferation of type II pneumocytes. Mitotic figures in II pneumocytes (arrows). Stained with toluidine blue. x 450

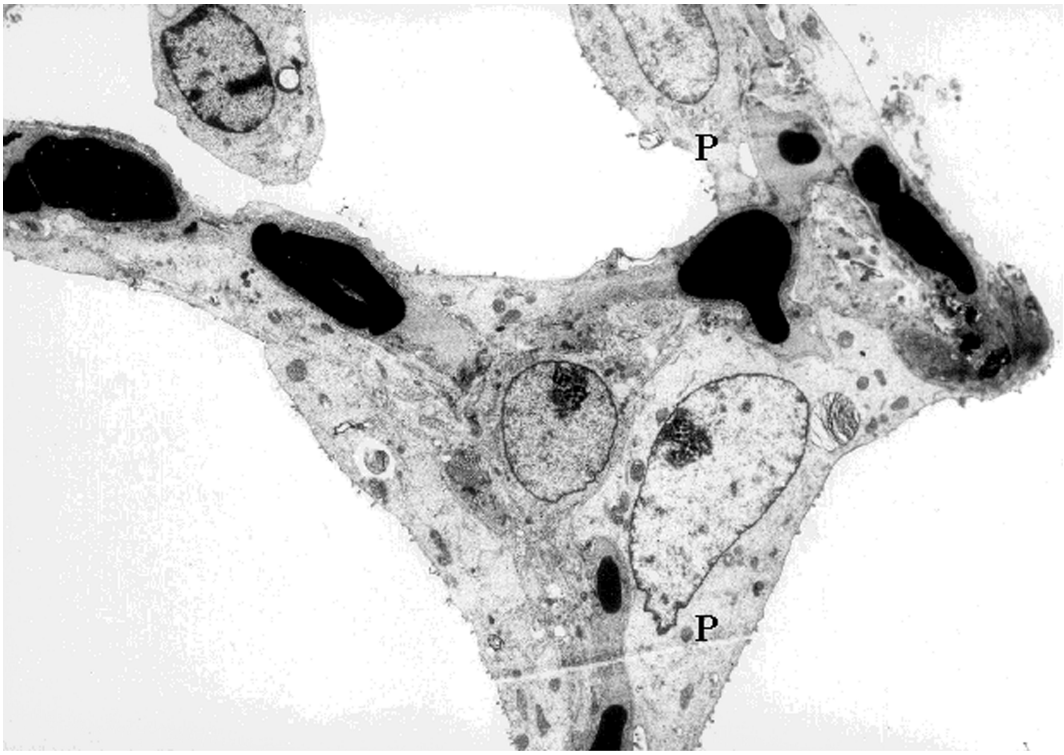

Fig. 7. Alveolar epithelium of rat 48 h after MMT injection. Numerous type II pneumocyte (P) young forms lining alveolar walls. x 3500

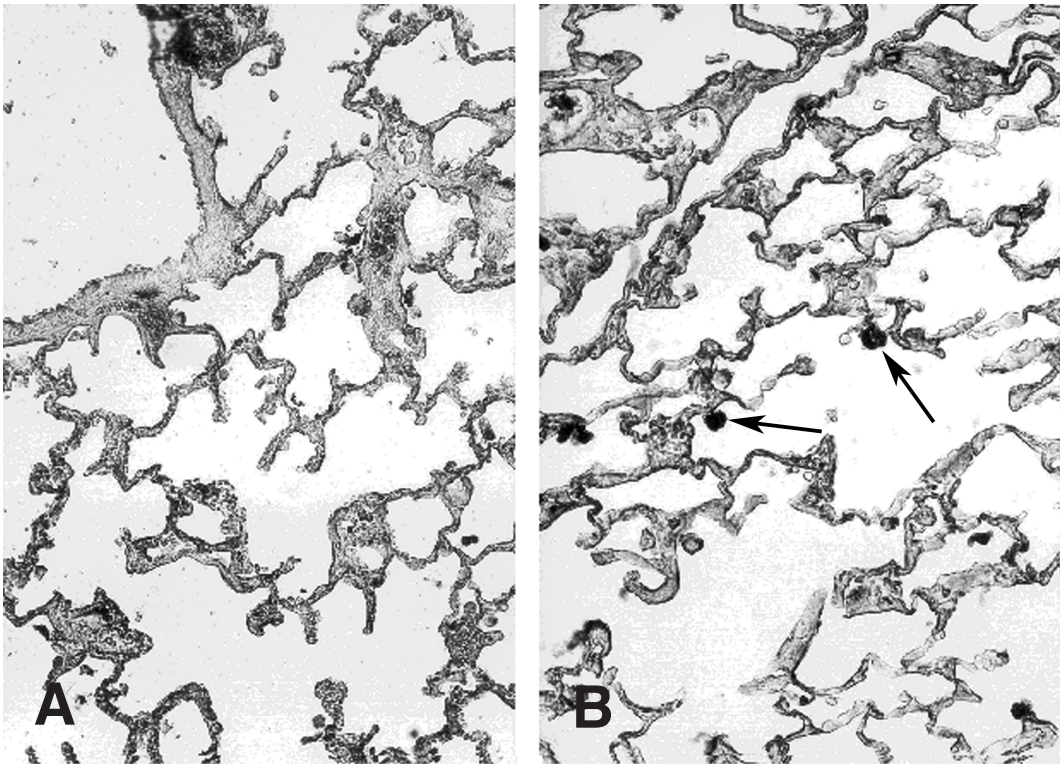

Fig. 8. Distribution of cell proliferation by immunodetection of BrdU incorporation (arrows) in alveoli of rat after MMT administration. Alveoli from corn oil treated controls **(A)** and MMT treated rats at 48 h PE **(B)**. x 70

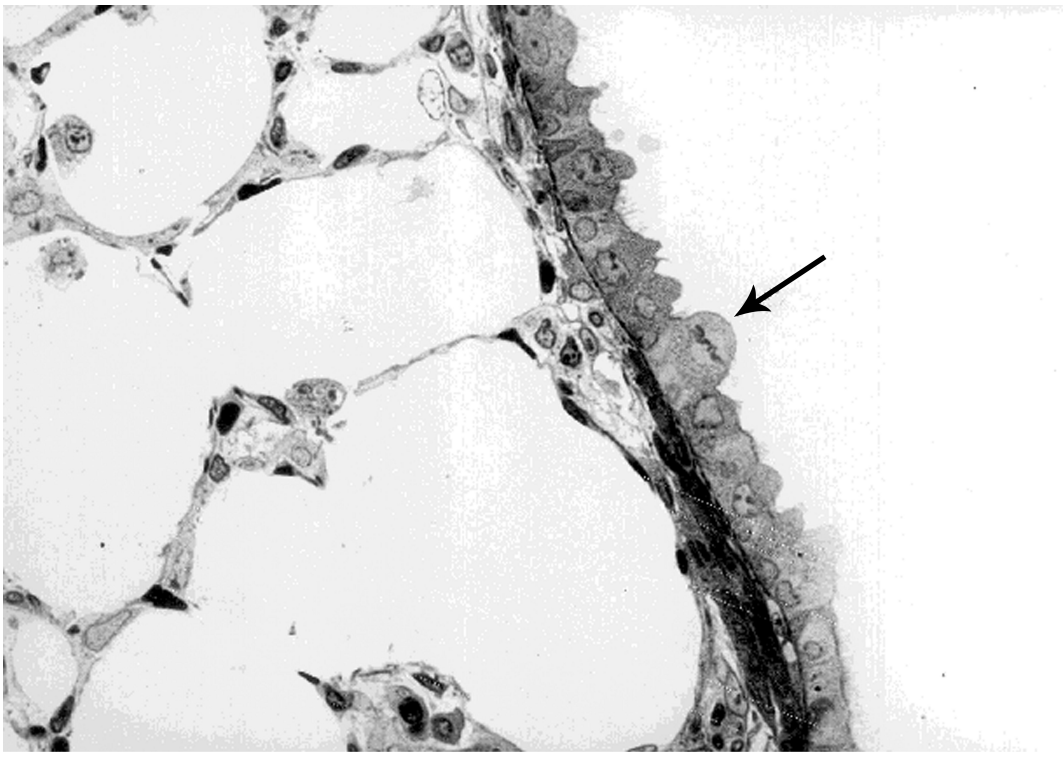

Fig. 9. Clara cell with mitotic figure (arrow) in bronchiolar epithelium of rat 48 h after MMT injection. Stained with toluidine blue. x 450

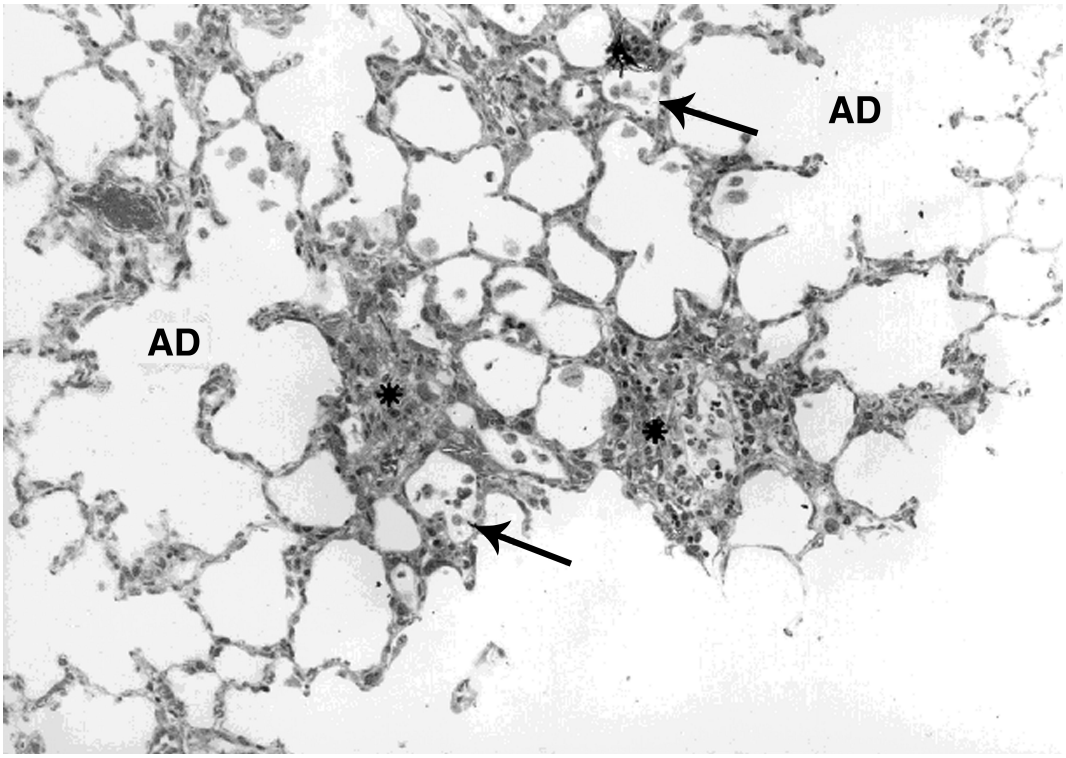

Fig. 10. Lung tissue of rat at seven days after MMT administration. Focal thickening of alveolar wall, primarily in the alveolar duct region (AD) and perivascular tissue. Some mononuclear cells (arrows) visible in the alveolar lumen. Stained with toluidine blue. x 200

and exceeded the values found for controls, but the difference was statistically insignificant. The concentration of CC16 protein in serum increased after exposure and at 48 h PE was significantly higher ($p<0.05$) than the values in the control group.

At 24 h PE, the concentration of total protein in BALF of MMT-exposed rats was 5 times as high as in the control group of rats not exposed to MMT. This effect of MMT exposure was even more pronounced 48 h PE and could be observed at seven days PE. The results of hyaluronic acid (HA) determination in BALF showed a significant rise in HA levels 24 h and 48 h PE (Table 1, Fig. 1). MMT exposure resulted in an increase in lung weight expressed as lung/100 g b.w. at 24 h, 48 h and 7 days after MMT administration compared to the findings for controls and the values at 12 h PE (Table 1, Fig. 1). The initial body weight in both groups: MMT-treated and controls, was similar and did not change throughout the whole experiment, data not shown.

Figure 2 shows EM micrograph of alveolar epithelium with type I and type II pneumocytes of control rat. Electron microscopic evaluation (Fig. 3) revealed that 12 h PE, the lung tissue of exposed rats was morphologically similar to that observed in the control animals. However, ultrastructural findings for the parenchymatous cells revealed vacuoles and swollen cytoplasm in some type I pneumocytes. Bronchiolar epithelium cells in most cases were not changed, but in some Clara cells, the dilation of SER intracellular membranes could be seen. Some of the ciliated cells had swollen mitochondria.

In the lungs of rats examined 24 h after MMT injection, alterations in type I pneumocytes were most prevalent. In many of these cells, the cytoplasm was severely swollen and cellular membranes were disrupted (Fig. 4). In the alveolar lumen, erythrocytes, neutrophils and macrophages were sometime present. In the Clara cells with dilated SER membranes and degenerative cytoplasm, vacuolation was found in the bronchiolar epithelium (Fig. 5). Some type II pneumocytes had vacuoles in their cytoplasm as well. At 48 h after MMT administration to rats, type II pneumocytes in mitotic phase could be observed. Proliferation of type II pneumocytes was confirmed by light microscopy (Fig. 6) and electron microscopy (Fig. 7) and by the presence of numerous type II pneumocytes with a BrdU-positive reaction (Fig. 8). Cell division, to a lesser extent, was noted also in Clara cells (Fig. 9). Destruction of cell membranes was still observed in type I pneumocytes.

Rats pulmonary tissue examination, seven days after MMT administration showed focal thickening of the alveolar wall, primarily in the alveolar duct region. Numerous mononuclear cells were found in the alveolar lumen (Fig. 10). EM analysis revealed numerous fibroblasts, macrophages, collagen and elastin fibres in the alveolar walls as well as alveoli macrophages and lymphocytes in the alveolar lumen. Type II pneumocytes were less numerous as compared with the findings at 48 h PE.

Discussion

Several countries, including Canada, the United States, Argentina, Australia, Bulgaria, France and Russia have replaced lead (Pb) in gasoline with the manganese-containing antiknock compound MMT (Zayed, 2001). It has been well documented that MMT, regardless of the route of administration, can cause organ-specific, pulmonary effects (Hanzlik et al., 1980; Hakkinen and Haschek, 1982; Haschek et al., 1982; MacGinley et al., 1987; Clay and Morris, 1989). This study confirmed that in MMT toxicity, rat alveolar and bronchiolar epithelium was the target. The process of lung injury was dynamic and consisted of several steps. The earliest changes in ultrastructure were observed in type I pneumocytes (Fig. 3) and in bronchiolar epithelium Clara cells 12 h PE. Degenerative changes in the epithelium, consisting of cytoplasmic vacuolation and disruption of cellular membranes in rat type I pneumocytes could be found 24 h PE. (Fig. 4). The damage of the blood-air barrier was manifested by an increase in total protein in BALF and increased levels of CC16 in serum (Table 1, Fig. 1). In Clara cells, mild degenerative changes were observed at the same time and were confirmed by decreased levels of CC16 protein in BALF. As an index of toxicity, increased level of total protein in BALF was noted at 24 h PE (Figs. 1 and 2). Decreased ratio of CC16 to total protein in BALF could be found at 24 h, 48 h and 7 days; however seven days PE, the CC16 level returned to control values (Table 1, Fig. 1). These changes reflect a massive leakage of proteins from the blood vessels into the alveolar space due to the alteration in permeability of blood vessels/bronchoalveolar barrier (Jones et al., 1983; Broeckaert et al., 2000). The proliferation of type II pneumocytes (Figs. 6, 7), and to a lesser extent of Clara cells, as seen 48 h after MMT i.p. injection, was evidenced by the presence of mitotic-phase cells and cells incorporating BrdU into their DNA (Fig. 8). These observations confirmed that type II pneumocytes participated in the regeneration of disrupted epithelium (Dormans, 1996). It seems reasonable to suppose that Clara cells also show the progenitor activity (Van Winkle et al., 1999). The role of the Clara cell in the renewal of the bronchiolar epithelium in the rat was well defined in some investigations (Evans et al., 1978; Harard et al., 1996).

The reported impairment and massive proliferation of type II pneumocytes and milder effects in Clara cells may be due to the different antioxidant reserves in these cells. A key protective system present in the lung is gluthatione with associated enzymes (Meister and Anderson, 1983; Cantin and Begin, 1991). The reaction of glutathione with electrophiles is catalysed by glutathione S-transerases, a family of closely related enzymes, several of which have been shown to be present in lung tissue. The GSTs in the lung tend to be bronchiolar in disposition. The activation of GSH-S-transferase was recognized as another class of secondary antioxidant mechanisms (Aniya and Naito, 1993). The

increase in GST activity observed 12 h and 24 h after MMT administration (Table 1) suggests an induction of oxidative stress in lung cells (Bouttin et al., 1998; Monteil et al., 1999; Shukla et al., 2000). It was shown that MMT metabolites were present in the lungs as soon as 3 h after MMT exposure (McGinley et al., 1987). In the study by Cox et al. (1987), MMT was found to have an LD50 value of 12.1 mg/kg. Neither of the metabolites appeared to have significant acute toxicity even when doses as high as 250 mg/kg had been given. The difference in toxicity may be due in part to the changes in the solubility of MMT metabolites (Zheng et al., 2000). This implies that the oxidative metabolism of MMT that results in the formation of these metabolites is an important detoxifying pathway.

Clara cells and type II pneumocytes are the principal source of CYP450 (CYP 1A1) monooxygenase activity in the lung (Boyd, 1977; Jones et al., 1983; Plopper et al., 1992, Verschoyle et al., 1993; Blanchard et al., 1996). The increase in GSH-S-transferase activity observed in our study suggests that MMT toxicity can be related to P450-dependent monoooxygenase MMT metabolites. SER dilation noted 12 h after MMT injection in metabolically active Clara cells supports these observations. The rat was considerably more sensitive to MMT than the mouse (Hakkinen and Haschek, 1982), may be because much lower GST levels were demonstrated in the rat Clara cells (Mainwaring et al., 1996). In oxidative stress, regardless of the mechanism involved in its generation (e.g. via oxygen radicals, redox cycling, lipid peroxidation, GSH depletion), an increase in GSH-S-transferase activity is an important factor (Sagara et al., 1998).

Ultrastructural examination of swollen SER membranes in Clara cells supports these observations (Fig. 4). As suggested earlier, an activation of organomanganese (MMT) compounds in Clara cells probably leads to the production of active metabolites which are capable of reaching the target in the rat type II pneumocyte (Clay and Morris, 1989; Blanchard et al., 1996). The GSTs appear to behave as reservoirs for both GSH conjugates and nonsubstrate ligands, presumably releasing them slowly for further metabolism in the cell. The most abundant targets for GSTs are polyunsaturated fatty acyl residues in phospholipids. GSH-S-transferase may be activated by oxygen radicals via forming dimmer protein with an S-S bond and can act as selenium-independent peroxide/scavenging lipid peroxides (Aniya and Naito, 1993). Free fatty acids can be made available for GSTs by action of phospholipase A2 (Tan et al., 1984; Van Kuijk et al., 1987). Clara cells protein (CC16) as a potent inhibitor of phospholipase A2 may play a role in the control of repair of membrane damage and in an anti-inflammatory action (Jorens et al., 1995). Based on the findings that CC16 is deficient in fibrotic lung diseases and that decreased CC16 levels well correlate with enhanced PLA2 activity in alveolar fluids, it was speculated that CC16 deficiency might contribute, by loss of regulatory control, to triggering the cascade leading to fibroblast activation and overgrowth (Lesur et al., 1995).

Increased HA level in BALF of intoxicated rats was observed 24 h and 48 h after MMT administration (Table 1). The excess synthesis of HA may be a consequence of fibroblast proliferation. Hyaluronic acid may take part in tissue remodelling during inflammation and repair, and may originate from interstitial fibroblasts (Cooper and Rathbone 1990). Increased levels of HA in BALF have been reported in experimental models, e.g. bleomycin-induced alveolitis in the rat (Nettenblad et al., 1989) or in glutaraldehyde exposure (Halatek et al., 2003). These findings indicate that MMT-induced inflammation is associated with changes in the production/degradation of extracellular matrix components (Teder and Heldin, 1997).

It is possible that HA is produced by the lung in response to complement-mediated stimulation. The results of the studies discussed above support the possible mechanism of pulmonary fibrosis with epithelial repair and subsequent proliferation of fibroblasts (Hashek and Witschi, 1979; Dunsmore et al., 1996; Sannes et al., 1996). The fibrotic process may have a close relationship with enhanced HA level observed in the present study after 24 and 48 h, and may by related to fibrosis observed after seven days. At that time-point, the HA level in BALF decreased and numerous fibroblasts, macrophages, collagen and elastin fibres, indicating an advanced repair process, were found in the lungs. However, the high level of total protein indicated a still enhanced permeability of blood/bronchoalveolar barrier (Table 1, Fig. 1). It was reported that in rats, the epithelial cells of bronchioles returned to normal 21 days after MMT treatment (Hakkinen and Haschek, 1982).

In conclusion, the injury and repair of bronchial epithelium cells, especially of Clara cells and type II pneumocyte cells, play an important part in MMT toxicity, probably depending on the antioxidant status of these cells. The sensitive biomarkers of CC16 and hyaluronic acid in BALF and serum reflect lung injury and indicate the time course of pulmonary damage and repair processes.

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References

Aniya Y. and Naito A. (1993). Oxidative stress-induced activation of microsomal glutathione S-transferase in isolated rat liver. Biochem. Pharmacol. 45, 37-42.

Bernard A., Marchandise F.X., Depelchin S., Lauwerys R. and Sibille Y. (1992). Clara cell protein in serum and bronchoalveolar lavage. Eur. Resp. J. 5, 1231-1238.

Bjermer L., Engstrom-Laurent A., Lundgren R., Rosenhall L. and

Hallgren R. (1987). Hyaluronate and type III procollagen peptide concentrations in bronchoalveolar lavage fluid as markers of disease activity in farmer's lung. Br. Med. J. Clin. Res. 295, 803-806.

Blanchard K.T., Clay R.J. and Morris J.B. (1996). Pulmonary activation and toxicity of Cyclopentadienyl Manganese Tricarbonyl. Toxicol. Appl. Pharmacol. 136, 280-288.

Blaschke E., Eklund A. and Hernbrand R. (1990). Determination of fibronectin and its degradation products in bronchoalveolar lavage fluid. Scand. J. Clin. Lab. Invest. 50, 619-626.

Boutin A-C., Shirali P., Garcon G., Gosset P., Leleu B., Marez T., Bernard A. and Haguenoer J-M. (1998). Peripherial markers (Clara cell protein and α-glutathione S-transferase) and lipoperoxidation (malondialdehyde) assessment in Sprague-Dawley rats instilled with haematite and Benzo[a]pyrene. J. Appl. Toxicol. 18, 39-45.

Boyd M.R. and Reznik-Schuller H.M. (1984). Metabolic basis for the pulmonary Clara cell as a target for pulmonary carcinogenesis. Toxicol. Pathol. 12, 56-61.

Boyd M.R. (1977). Evidence for the Clara cell as a site of cytochrome P-450-dependent mixed-function oxidase activity in lung. Nature 269, 713-715.

Broeckaert F., Arsalane K., Hermans C., Bergamaschi E., Brustolin A., Muttii A. and Bernard A. (2000). Srum Clara cell protein: A sensitive biomarker of increased lung epithelium permeability caused by ambient ozone. Environ. Health Perspect. 108, 533-537.

Cantin A.M. and Begin R. (1991). Glutathione and inflamatory disorders of the lung. Lung 169, 123-138.

Clay R.J. and Morris J.B. (1989). Comparative pneumotoxicity of Cyclopentadienyl Manganese Tricarbonyl and Methylcyclopentadienyl Manganese Tricarbonyl. Toxicol. Appl. Pharmacol. 98, 434-443.

Cooper E.H. and Rathbone B.J. (1990). Clinical significance of the immunometric measurements of hyaluronic acid. Ann. Clin. Biochem. 27, 444-451.

Cox D.N., Traiger G.J., Jacober S.P. and Hanzlik R.P. (1987). Comparison of the toxicity of methylcyclopentadienyl manganese tricarbonyl with that of its two major metabolites. Toxicol. Lett. 39, 1-5.

Davidson J.M. (1990). Biochemistry and turnover of lung interstitium. Eur. Respir. J. 3, 1048-1068.

Dunsmore S.E., Lee Y.C., Martinez-Williams C. and Rannels E. (1996). Synthesis of fibronectin and laminin by type II pulmonary epithelial cells. Am. J. Physiol. 270. (Lung Cell. Mol. Physiol 14) L215-L223.

Durham S.K. and Gijbels M.J. (1989). Long-term morphologic and biochemical changes induced by O,O,S-Trimethyl Phosphorothiolate in rat lung. Toxicol. Appl. Pharmacol. 98, 465-474.

Engstrom-Laurent A., Laurent U.B.G., Lilij K. and Laurent T. (1985). Concentration of sodium hyaluronate in serum. Scand. J. Clin. Lab. Invest. 45, 497-504.

Evans M.J., Cabral-Anderson L.J. and Freeman G. (1978). Role of the Clara cell in the renewal of the terminal bronchiolar epithelium in the rat. Lab. Invest. 38, 648-655.

Forkert P.G. (1985). Bromobenzene causes Clara cell damage in mice. Can. J. Physiol. Pharmacol. 63, 1480-1484.

Habig W., Pabst M. and Jakoby W. (1974). Glutatione S-transferase: the first enzymatic step in mercapturic acid formation. J. Biol. Chem, 249, 130-7139.

Hackett B.P., Shimizu N. and Gitlin J.D. (1992). Clara cell secretory protein gene expression in bronchiolar epithelium. Am. J. Physiol. 262, L399-404.

Hakkinen R.P. and Haschek W.M. (1982). Pulmonary toxicity of methylcyclopentadienyl manganese tricarbonyl: nonciliated bronchiolar epithelial (Clara) cell necrosis and alveolar damage in the mouse, rat, and hamster. Toxicol. Appl. Pharmacol. 65, 11-22.

Halatek T., Hermans C., Wattier R., Falamagne P., Toubeau G. and Bernard A. (1998). Quantification of Clara cell protein in rat and mouse biological fluids using a sensitive immunoassay. Eur. Resp. J. 11, 726-733.

Halatek T., Opalska B., Swiercz R., Palczynski C., Górski P., Rydzynski K. and Bernard A (2003). Glutaraldehyde inhalation exposure of rats: Effects on morphology of the lungs and Clara cell protein and hyaluronic acid levels in BAL. Inhal. Toxicol. 15, 85-97.

Hallgren R., Eklund A., Engstrom-Laurent A. and Schmekel B. (1985). Hyaluronate in bronchoalveolar lavage fluid: a new marker in sarcoidosis reflecting pulmonary disease. Br. Med. J. 290, 1778-1781.

Hanzlik R.P., Stitt R. and Traiger G.J. (1980). Toxic effects of Methylcyclopentadienyl Manganese Tricarbonyl (MMT) in rats: Role of metabolism. Toxicol. Appl. Pharmacol. 56, 353-360.

Harard A-L., Zahm J-M., Pierrot D., Hinnrasky J., Fuchey C. and Puchelle E. (1996). Epithelial barrier integrity during in vitro wound repair of the airway epithelium. Am. J. Respir. Cell. Mol. Biol. 5, 624-632.

Haschek W.M., Hakkinen P.J., Witschi H.P., Hanzlik R.P. and Traiger G.J. (1982). Nonciliated bronchiolar epithelial (Clara) cell necrosis induced by organometallic carbonyl compounds. Toxicol. Lett. 14, 85-92.

Haschek W. and Witschi H. (1979). Pulmonary fibrosis - a possible mechanism. Toxicol. Appl. Pharmacol. 51, 475-487.

Hinderer R.K. (1979). Toxicity studies of methylcyclopentadienyl manganese tricarbonyl (MMT). Amer. Ind. Hyg. Assoc. J. 40, 164-167.

Jones J.G., Royston, D. and Minty B.D. (1983). Changes in alveolar-capillary barrier function in animals and humans. Am. Rev. Respir. Dis. 127, S51-S59.

Jones K.G. Holland J.F., Foureman G.L., Bend J.R. and Fouts J.R. (1983). Xenobiotic metabolism in Clara cells and in alveolar type II cells isolated from rats treated with ß-naphtaflavone. J. Pharmacol. Exp. Ther. 225, 316-319.

Jorens Ph.G., Sibille Y., Gouldin N.J., Van Overveld F.J., Herman A.G., Bossaert L., De Backer W.A., Lauwerys R., Flower R.J. and Bernard A. (1995). Potential role of Clara cell protein, an endogenous phospholipase A2 inhibitor, in acute lung injury. Eur. Resp. J. 8, 1647-1653.

Lesur O., Bernard A., Arsalane K., Lauwerys R., Bégin R., Cantin A. and Lane D. (1995). Clara cell protein (CC16) induces a phospholipase A2-mediated inhibition of fibroblast migration in vitro. Am. J. Resp. Crit. Care Med. 152, 290-297.

Lin C.Y., Isbell M.A., Morin D., Boland B.C., Salemi M.R., Jewell W.T., Weir A.J., Fanucchi M.V., Baker G.L., Plopper C.G. and Buckpitt A.R. (2005). Characterization of a structurally intact in situ lung model and comparison of naphthalene protein adducts generated in this model vs lung microsomes. Chem. Res. Toxicol. 218, 802-813.

Mainwaring G.W., Williams S.M., Foster J.R., Tugwood J. and Green T. (1996). Distribution of Theta-class glutathione S-transferases in the liver and lung of mouse, rat and humans. Biochem. J. 318, 297-303.

Mantile C., Miele L., Cordella-Miele E., Singh G., Katyal S. and Mukherjee A.B. (1993). Human Clara cell 10 KDa protein is the counterpart of rabbit uteroglobin. J. Biol. Chem. 268, 20343-20351.

Meister A. and Anderson M.E. (1983). Glutathione. Annu. Rev. Biochem. 52, 711-760.

McGinley P.A., Morris J.B., Clay R.J. and Gianutsos G. (1987). Disposition and toxicity of methylcyclopentadienyl manganese tricarbonyl in the rat. Toxicol. Lett. 366, 137-145.

Miele L., Cordella-Miele E. and Mukherjee A.B. (1987). Uteroglobin: structure, molecular biology, and new perspectives on its function as a phospholipase A2 inhibitor. Endocrine Rev. 8, 474-490.

Monteil C., Le Prieur E., Buisson S., Morin J.P., Guerbet M. and Jouany J.M. (1999). Acrolein toxicity: comparative in vitro study with lung slices and pneumocytes type II cell line from rats. Toxicology 133,129-138.

Nettelbladt O., Bergh J., Schenholm M., Tengblad A. and Hallgren R. (1989). Accumulation of hyaluronic acid in the alveolar-interstitial tissue in bleomycin-induced alveolitis. Am. Rev. Respir. Dis. 139, 759-762.

Penney D.A., Hogberg K., Traiger G.J. and Hanzlik R.P. (1985). The acute toxicity of cyclopentadienyl manganese tricarbonyl in the rat. Toxicology 34, 341-347.

Plopper C.G., Macklin J., Nishio S.J., Hyde D.M. and Buckpitt A.R. (1992). Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. III Morphometric comparison of changes in the epithelial populations of terminal bronchioles and lobar bronchi in mice, hamsters, rats after parenteral administration of naphthalene. Lab. Invest. 67, 553-565.

Rennard S.I. and Crystal R.D. (1982). Fibronectin in human bronchoalveolar lavage fluid. Elevation in patients with interstitial lung disease. J. Clin. Invest. 69, 113-122.

Sagara Y., Dargusch R., Chambers D., Davis J., Schubert D. and Maher P. (1998). Cellular mechanisms of resistance to chronic oxidative stress. Free Radic. Biol. Med. 24, 1375-1389.

Sannes P.L., Khosla J. and Cheng P-W. (1996). Sulfation of extracellular matrices modifies responses of alveolar type II cells to fibroblast growth factors. Am. J. Physiol 271 (Lung Cell. Mol. Physiol 15), L688-L697.

Shukla G.S, Shukla A., Potts R.J., Osier M., Hart B.A. and Chiu J.F. (2000). Cadmium-mediated oxidative stress in alveolar epithelial cells induces the expression of gamma-glutamylcysteine synthetase catalytic subunit and glutathione S-transferase alpha and pi isoforms: potential role of activator protein-1. Cell Biol. Toxicol. 16, 347-362.

Sulkowska M., Sulkowski S. and Dzieciol J. (1996). Type II alveolar epithelial cells promote fibrosis during development of experimental lung emphysema. Folia Histochem. Cytobiol. 34, 27-28.

Tan K.H., Meyer D.J. and Ketterer B. (1984). Inhibition of microsomal lipid peroxidation by glutathione and glutathione transferases B and AA. Role of endogenous phospholipase A2. Biochem. J. 220, 243-252.

Teder P. and Heldin P. (1997). Mechanism of impaired local hyaluronan turnover in bleomycin-induced lung injury in rat. Am. J. Respir. Cell Mol. Biol. 17, 376-385.

Van Kuijk F.J.G.M, Sevanian A., Handelman G.J. and Dratz E.A. (1987). A new role for phospholipase A2: Protection of membranes from lipid peroxidation damage. Trends Biochem. 12, 31-34.

Van Winkle L.S., Buckpitt A.R., Nishio S.J., Isaac J.M. and Plopper G. (1995). Cellular response in naphthalene-induced Clara cell injury and bronchiolar epithelial repair in mice. Am. J. Physiol 269 (Lung Cell. Mol. Physiol 13), L800-L818.

Van Winkle L.S., Johnson A.A. Nishio S.J., Isaac J.M., Brown C.D. and Plopper G. (1999). Early events in naphtalene-iduced acute Clara cell toxicity. Am. J. Respir. Cell Mol. Biol. 21, 44-53.

Verschoyle R.D., Wolf C.R. and Dinsdale D. (1993). CYP2B1 is responsible for the pulmonary bioactivation and toxicity of butylated hydroxytoluene, O,O,S-trimethylphosphothiotate and methylcyclopentadienyl manganese tricarbonyl. J. Pharmacol. Exp. Ther. 226, 958-963.

Woods L.W., Wilson D.W., Schiedt M.J. and Giri S.N. (1993). Structural and biochemical changes in lungs of 3-methylindole-treated rats. Am. J. Pathol. 142, 129-138.

Zayed J. (2001). Use of MMT in Canadian gasoline. Health and environmental issues. Am. J. Ind Med. 39, 426-433.

Zheng W., Kim H. and Zhao Q. (2000). Comparative toxicokinetics of manganese chloride and methylcyclopentadienyl manganese tricarbonyl (MMT) in Sprague-Dowley rats. Toxicol. Sciences 54, 295-301.

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