

Inflammatory and cytotoxic effects as well as histological findings of selected industrial fibrous dusts in Fischer rats after intratracheal instillation

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The problem of the effects of industrial fibrous dusts on the respiratory tract remains topical. The mechanism of action of fibrous structures during lung disease development is not clear up to the present time. With the aim of understanding better the mechanism of lung injury after a low fibres dose exposure we investigated some inflammatory and cytotoxic bronchoalveolar lavage parameters (number of leukocytes and alveolar macrophages; differential cell count; total amount of protein; phagocytic activity and viability of alveolar macrophages; activity of lactate dehydrogenase, acid phosphatase and alkaline phosphatase; and histology of lungs) 4 and 16 weeks after intratracheal instillation in rats of 2 mg amosite – asbestos and wollastonite (an asbestos substitute) fibres. The main conclusions of the study were as follows. Following treatment with amosite fibres, time-dependent adverse effects were observed, with most of the evaluated bronchoalveolar lavage parameters being significantly changed in comparison with relevant controls. Amosite gave rise to very definite, fibrosing chronic inflammation by the end of 4 weeks, while progressive fibrosis with the obliteration of the lumina of bronchioli and alveoli could be seen after 16 weeks of exposure. In contrast to these observation, treatment with wollastonite fibres very weakly influenced bronchoalveolar lavage parameters and lung structures in comparison with control animals. Very minimal fibrosing alveolitis with moderate progression was found by the end of the 16 weeks. Based on these *in vivo* observation, it is concluded that wollastonite fibres seem to be much less toxic and fibrogenic than asbestos fibres.

Key words: fibrous dusts, amosite, wollastonite, bronchoalveolar lavage, inflammation, cytotoxicity, histological findings.

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Introduction

The potential effects of industrial fibrous dusts on the respiratory system represent a potential occupational and environmental health hazard for humans. It is known that prolonged exposure to asbestos can cause pleural plaques, asbestosis and malignant tumours (BUCHANOVÁ et al., 1988; HURBÁNKOVÁ & KAIGLOVÁ, 1993; MORINAGA, 1993; HILLERDAL & HENDERSON, 1997). Consequently efforts are ongoing to substitute such fibres with fibres that, while technologically comparable to asbestos, have lower toxicity. This task requires extensive research activities to better understand the physiological and toxicological effects of fibre substitutes. These activities are in line with World Health Organisation, International Labour Office and International Agency For Research On Cancer guidelines (Environm. Health Criteria 77, 1988; IARC Monographs, MMMF and Radon, 1988; Executive Summary of a WHO Consultation, 1992; IARC Monographs, Silica, some silicates, coal dust and para-aramid fibrils, 1997; IARC Monographs, Man-made vitreous fibres, 2002). The mechanism by which asbestos and other fibrous dusts cause lung diseases has not been fully explained yet. It seems that type of fibres, dose, duration of exposure and individual immunodisposition play an important role in the induction of lung tissue injury. Numerous studies suggest that the process of lung injury after asbestos exposure depends besides other factors also on the dimensions of the fibres (KOHYAMA et al., 1997; HURBÁNKOVÁ et al., 1998; GREIM et al., 2001). Some naturally occurring or man-made substitutes of asbestos would be judged as pathogenic according to STANTON's hypothesis, which states that long, thin particles are associated with lung diseases, the most pathogenic being fibres with diameter less than $0,25 \mu\text{m}$ and length longer than $8 \mu\text{m}$ (STANTON et al., 1981). The aim of the present study was to examine and compare the effects of dose (2 mg) of wollastonite (a possible asbestos substitute fibrous material) and amosite asbestos, 4 or 16 weeks after intratracheal instillation (i.t.) in rats.

Material and methods

The study was conducted with the approval of the Animal Ethics Committee of the Institute of Preventive and Clinical Medicine (Bratislava, Slovakia) and in accordance with the guidelines of European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes. Male Fisher 344 rat were supplied by Charles River Company, Germany. At the

beginning of instillation – after two weeks quarantine – mean initial weight of animals was 165–170 g. All animals were maintained under non-infectious laboratory conditions at $22 \pm 2^\circ\text{C}$, 45% relative humidity, under natural light and normal light/dark photoperiodicity and under air-conditioned conditions using the unit WOLF KG 100 (WOLF – Clima Technik, GmbH, Mainburg, Germany). A commercial diet ST 1 (TOP – Dovo, Horné Dubové, Slovakia) and tap water were available *ad libitum*. The fibres used in our experiments were amosite (a naturally occurring silicate of the amphibole asbestos group) and wollastonite (a naturally occurring silicate used as a substitute of asbestos in some industrial applications).

Animals (5–8 per group) were intratracheally instilled (i.e. non-invasively) under light ether anaesthesia, with amosite or wollastonite fibre suspension (2 mg suspended in 0.2 mL of saline solution per animal) or only with 0.2 mL saline per animal (control group) for investigation of bronchoalveolar lavage (BAL) parameters. Moreover, five animals were treated for histology and three animals were treated for electron microscopy.

Bronchoalveolar lavage studies

Four or sixteen weeks after intratracheal instillation the animals were sacrificed by severing the abdominal aorta under intra peritoneal thiopental narcosis (150 mg/kg of animal). AM were harvested using a modified method of BAL by MYRVIK et al. (1961). The trachea was cannulated, and the lungs were washed 3 times with 4 mL of saline solution (*in situ*). The cell number per mL BAL fluid was determined in Burker's chamber and differential cell counts were performed on May-Grunwald-Giemsa stained preparations. The BAL-fluid obtained was centrifuged at $500 \times g$ for 10 min at 4°C . In the cell-free supernatant (the cell-free BAL fluid) the activities of lactate dehydrogenase (LDH), acid phosphatase (AcP) and alkaline phosphatase (AP) were determined. The BAL cell sediment was re-suspended to a concentration of 10^6 AM/mL and immediately used for phagocytic activity determination. For determination of AcP activity in BAL cells, the cell suspension was re-suspended in Triton-PBS (final concentration of Triton: 0.1%), frozen and thawed three times and finally centrifuged at $14,000 \times g$ for 15 min. The activity of AcP was measured in the supernatant.

The phagocytic activity of AM was measured by the method of FURNUSEK et al. (1982) using 2-hydroxyethylmetacrylate particles (HEMA, Neosys, Prague, Czech Republic). Fifty mL of HEMA particles in PBS were added to 100 μL of BAL fluid and incubated for 60 min at 37°C with shaking at frequent intervals. Staining was performed by the May-Grunwald Giemsa method. Cells were considered positive when they phagocytized three or more particles (FURNUSEK et al., 1982). Differential cell counts were performed on May-Grunwald Giemsa stained preparations (% Gr, AM and Ly in 200 cells).

For the examination of the viability of AM, 200 μL of 0.25% erythrosine solution were added to a 200 μL aliquot of the cell suspension. The numbers

of viable and non-viable cells were counted using a Bürker's chamber.

LDH, AcP and AP activities were determined spectrophotometrically using LD-105 UV kits (LA-CHEMA Brno, Czech Republic) for LD, AC 565 kits – RANDOX (Randox Laboratories, UK) for AcP and according to Morton modified by KOVÁČIKOVA (1990) for AP, respectively. Protein was measured by the Lowry method, the bovine serum albumin being used as standard for the calibration curve (LOWRY et al., 1951).

The following BAL parameters were examined:

Inflammatory response biomarkers

- number of leukocytes (Le)/mL BAL fluid
- number of AM/mL BAL fluid
- differential cell count [AM, lymphocytes (Ly), granulocytes (Gr)]
- total amount of protein

Cytotoxic parameters

- phagocytic activity of AM
- viability of AM
- LDH activity (in the cell-free lavage fluid)
- AcP activity (in the cell-free lavage fluid and in the BAL cell suspension)
- activity of AP (in the cell-free lavage fluid)

The results were statistically evaluated using Wilcoxon's test.

Morphology

Light microscopy. Animals were sacrificed after 4 or 16 weeks of exposure, lungs and regional lymph nodes were fixed in 8% phosphate buffered neutral formalin (pH 7.4), after dehydrated with alcohol, embedded in paraffin and celloidine and stained with hematoxylin-eosin, Gömöri's silver impregnation (5–7 μm sections). We studied the membranes of pneumocytes by lectin histochemistry: Ricinus Communis Agglutinin (RCA) for type I pneumocytes and Maclura Pomifera Agglutinin (MPA) for type II pneumocytes. Biotin labelled RCA and MPA (Sigma, St. Louis, MO, USA) were used. In brief, endogenous peroxidase activity was destroyed by a 30-min treatment with 0.3% H_2O_2 in TBS (Tris-buffered saline, pH 7.4). Sections (4–5 μm) were rinsed in TBS, covered with bovine serum albumin (Sigma) and washing with TBS for 10 min then incubated in biotin labelled RCA (20 $\mu\text{g}/\text{mL}$) and biotin labelled MPA (30 $\mu\text{g}/\text{mL}$) for 1 h. Peroxidase activity was visualized with 0.05% 3,3'-diaminobenzidine HCL and 0.015 H_2O_2 (Dobbs et al., 1980; 1985)

Transmission electron microscopy. Lungs (from one control and three treated animals 16 weeks after treatment) were perfused by 2% buffered glutaraldehyde (pH 7.4) through a canula inserted in the trachea. Small lungs specimens (1 mm^3) were post-fixed in 1% osmic acid and embedded in Durcupan ACM Fluka after dehydration with ethanol and propylene oxide. Ultrathin sections were cut (Ultracut, Reichert), stained with uranyl acetate and lead citrate and investigated with a JEOL JEM 100C transmission electron microscope.

Table 1. The length and diameter of amosite fibres.

Length (μm)	Fibres %	Mean diameter (μm)
< 20	5	0.71
20–30	75	
>30	20	

Table 2. The length and diameter of wollastonite fibres.

Length (μm)	Fibres %	Mean diameter (μm)	Fibres
0–8	38.5	< 0.8	70
9–15	14.85	< 2.0	30
16–25	23.2		
26–35	15.58		
> 35	7.84		

Determination of fibre dimensions

The fibres were suspended in distilled water and examined by phase contrast microscopy at $\times 500$ magnification. The length and diameter of fibres are shown in Tables 1 and 2.

Results

The effects of fibre treatment on BAL parameters are described in Table 3 (4 week exposure) and Table 4 (16 week exposure).

Inflammatory response parameters

Wollastonite did not induce any significant changes in comparison with the corresponding control groups, at either exposure time, only a non-statistically significant trend to increase number of Le, % Ly (4 week exposure) and Gr being observed at both times. In contrast amosite caused significant increase in % of Ly and decrease in % of AM, compared with corresponding controls, at both exposure times. In addition, significant increases in number of Le (after 4 week exposure) as well as % of Gr (16 week exposure) were observed, respectively.

Cytotoxic parameters

Wollastonite significantly decreased viability of cells and increased LDH level but only 16 weeks after instillation. Amosite significantly increased level of LDH and decreased viability of AM in comparison with the relevant control at both ex-

Table 3. Inflammatory response and cytotoxic parameters in BAL (4 week exposure).^a

Parameters ^b	Groups of animals ^c			
	Wollastonite (2 mg/animal)		Amosite (2 mg/animal)	
	Control	Exposed	Control	Exposed
<i>n</i>	7	7	6	7
Number of Le (10 ³ · mL ⁻¹)	195 (149; 206)	223 (187.5; 255)	194 (147.5; 174.5)	277.5 (242.8; 296.3)*
Ly (% of BAL cells)	2 (1; 2)	2 (1; 2)	1 (0.5; 2)	4 (2; 5)**
AM (% of BAL cells)	97 (97; 99)	97 (96; 98)	98 (96.5; 98.5)	95 (93; 96)**
Gr (% of BAL cells)	1 (1; 1)	1 (1; 2)	1 (1; 1.5)	2 (1; 2)
Total amount of protein (mg)	1.19 (0.4; 2.27)	1.17 (1.05; 1.65)	1.78 (1.66; 1.90)	2.10 (1.61; 3.21)
Phagocytic activity of AM (%)	53 (46; 56.5)	48.5 (39; 57)	57 (43; 63.5)	45 (39.8; 52.8)
Viability (% of living cells)	88 (81; 91)	82 (80; 87)	90 (88; 90)	80 (77; 83)**
LDH (nkat.L ⁻¹)	0.76 (0.62; 1.30)	0.96 (0.64; 1.13)	0.66 (0.63; 0.75)	1.22 ±0.11**
AcP in cell-free BAL fluid (nkat · L ⁻¹)	16.48 (16.48; 19.30)	15.64 (13.14; 21.28)	13.99 (10.96; 14.52)	19 (12.814; 21.79)
AcP (nkat · 10 ⁻⁶ cells)	0.35 (0.18; 0.56)	0.39 (0.25; 0.89)	0.245 (0.24; 0.31)	0.307 (0.22; 0.32)
AP (μmol pNP · hr ⁻¹ · mg protein ⁻¹)	345.32 (257.1; 421.4)	270.54 (230.8; 341.7)	247.58 (216.0; 292.5)	232.69 (204.7; 286.5)

^a Values represent medians and 25th and 75th percentiles.

^b Abbreviations: BAL – bronchoalveolar lavage; Le – leukocytes; AM – alveolar macrophages; LDH – lactate dehydrogenase; AcP – acid phosphatase; AP – alkaline phosphatase; pNP – p-nitrophenol.

^c Group exposed to the fibrous dust in comparison with corresponding non-exposed control group.

* $p < 0.05$; ** $p < 0.01$.

posure times and decreased phagocytic activity 16 weeks after instillation. At least at 16 weeks post-exposure the whole differential count was significantly changed (significant increase in the % Gr and Ly, significant decreased in the % AM).

Histology

Light microscopy. There were no pathological changes in control lungs and regional lymph nodes in any periods examined. Wollastonite, 2 mg, 4 weeks after exposure: in the pulmonary interstitium, a very moderate, multifocal, chronic

Table 4. Inflammatory response and cytotoxic parameters in BAL (16 week exposure). ^a

Parameters ^b	Groups of animals ^c		
	Control	Wollastonite (2mg/animal)	Amosite (2mg/animal)
		Exposed	Exposed
<i>n</i>	7	8	8
Number of Le (10 ³ · ml ⁻¹)	250 (123.7; 147.5)	250 (215; 262.5)	230 (188.75; 265)
Ly (% of BAL cells)	2 (1; 3)	1 (1; 1)	4,5 (4; 6)**
AM (% of BAL cells)	97 (96; 98)	97.5 (97; 98)	91 (90.25; 93.5)***
Gr (% of BAL cells)	1 (1; 1)	1.5 (1; 2)	4 (3; 4)***
Total amount of protein (mg)	1.46 (1.12; 1.83)	1.24 (1.04; 1.40)	1.63 (1.22; 1.90)
Phagocytic activity of AM (%)	56 (48,5; 58.5)	56 (48; 57)	22 (17; 27)**
viability (% of living cells)	90 (86; 92)	85 (81.5; 86.75)*	81 (74.75; 84.75)**
LDH (nkat · L ⁻¹)	0,57 (0.53; 0.68)	0,75 (0.72; 0.99)*	1.24 (1.1; 1.45)**
AcP in cell-free BAL fluid (nkat · L ⁻¹)	8.6 (7.57; 9.92)	8.67 (7.42; 10.75)	8.35 (1.78; 10.86)
AcP (nkat · 10 ⁻⁶ cells)	0.14 (0.13; 0.29)	0.16 (0.14; 0.17)	0.25 (0.15; 0.32)
AP (μmol pNP · hr ⁻¹ · mg protein ⁻¹)	243.29 (219.3; 289.5)	273.94 (225.5; 277.2)	251.66 (211.7; 272.1)

^a Values represent medians and 25th and 75th percentiles.

^b Abbreviations: BAL – bronchoalveolar lavage; Le – leukocytes; AM – alveolar macrophages; LDH – lactate dehydrogenase; AcP – acid phosphatase; AP – alkaline phosphatase; pNP – p-nitrophenol.

^c Group exposed to the fibrous dust in comparison with the corresponding non-exposed control group.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

inflammatory response developed consisting of macrophages, Ly, plasma cells and few fibroblasts. The elastic network remained intact. In the inflammatory foci, the membranes of the alveolar epithelium did not show significant damage. In the in-

terstitium needle-like, positively birefringent wollastonite fibres could be detected between crossed polars.

Wollastonite, 2 mg, 16 weeks after exposure: The character of inflammation seen after 4 week

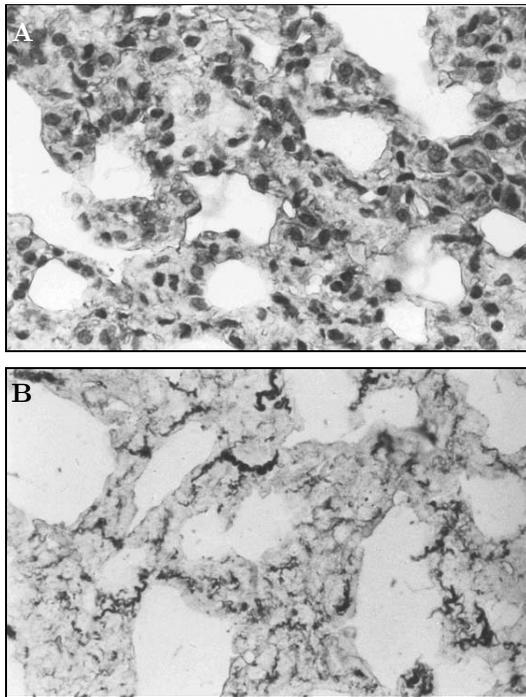


Fig. 1. Rat lung 16 weeks after treatment with a single intratracheal dose of 2 mg wollastonite per animal. (A) HE staining: in the pulmonary interstitium moderate chronic inflammation is seen. Magnification: $\times 650$. (B) Gömöri' silver impregnation: inside the inflammatory foci the amount of argyrophilic fibres increased but it is not significant. Magnification: $\times 650$.

exposure did not change (Fig. 1A). In the inflammatory foci, the amount of argyrophilic fibres showed a non significant increase (Fig. 1B). There were no detectable crystals in the lung. The regional lymph nodes, liver and spleen were without pathological changes.

Amosite, 2 mg, 4 weeks after exposure: Interstitially, subchronic inflammation appeared consisting of Ly, plasma cells, macrophages and fibroblasts. The interalveolar septa became thickened owing to an increase in the amount of collagen fibres, and the elastic network appeared fragmented within the inflammatory foci. In the interstitium needle-like positively birefringent crystals could be detected and some Berlin-blue positive bodies were observable. The regional lymph nodes, spleen and liver did not show any changes.

Amosite, 2 mg, 16 weeks after exposure: Very intense interstitial inflammation could be observed, consisting of Ly, plasma cells, macrophages and fibroblasts. The interalveolar septa were thickened, the lumina of bronchioli and alveoli became

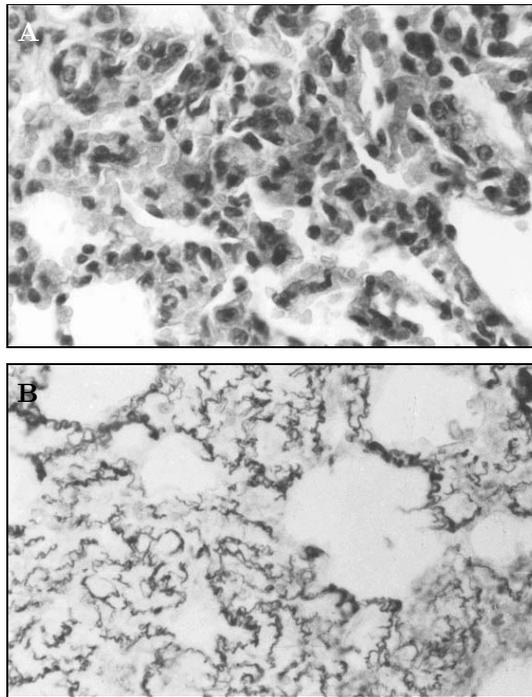


Fig. 2. Rat lung 16 weeks after treatment with a single intratracheal dose of 2 mg amosite per animal. (A) HE staining: the pulmonary interstitium became thickened and infiltrated with Ly, plasma cells, macrophages and fibroblasts. The majority of the lumina of the alveoli and bronchioli narrowed or obliterated. Magnification: $\times 650$. (B) Gömöri' silver impregnation: the argyrophilic fibres increased in the interstitium. Magnification: $\times 650$.

narrowed (Fig. 2A), and inside the inflammatory foci the amount of argyrophilic fibres increased irregularly (Fig. 2B).

The type I alveolar epithelial cells did not react with RCA within the inflammatory foci (Fig. 3A), whereas the type II cells were hypertrophic and showed increased reactivity with MPA (Fig. 3B). The elastic network completely disappeared. There were no changes in the regional lymph nodes, spleen and liver.

Transmission electron microscopy (after 16 week exposure only)

Following treatment with wollastonite, in the interalveolar septa, collagen fibrils could be observed under the alveolar epithelium (Fig. 4). After amosite treatment, type II pneumocytes were flattened and lost the majority of their microvilli and became similar to stratified epithelium (Fig. 5).

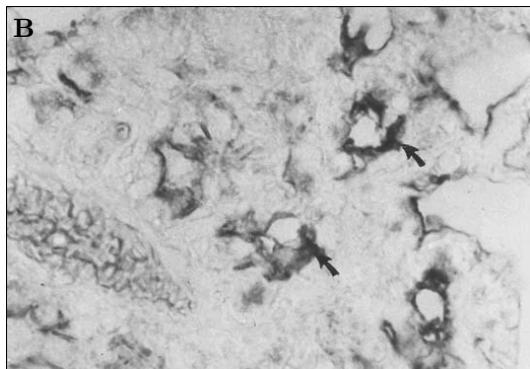
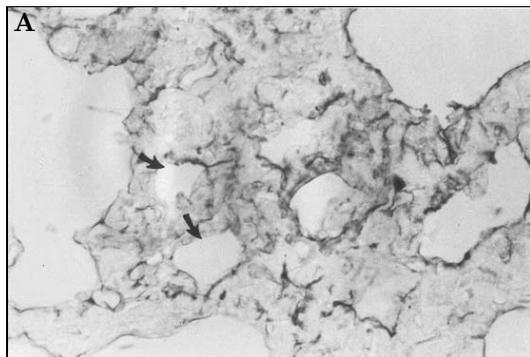


Fig. 3. Rat lung 16 weeks after treatment with a single intratracheal dose of 2 mg amosite per animal. (A) Reaction with RCA I: inside the inflammatory area the type I alveolar cells do not react with RCA (arrows). Magnification: $\times 650$. (B) Reaction with MPA: reaction became more extended owing to the hypertrophy of type II pneumocytes (arrows). Magnification: $\times 650$.

Discussion

Examination of the number and type of cells obtained via BAL as well as of their viability and state of activation, enables us to understand the extent of the harmful effects caused in the lung by inhaled noxious substances. Inhalation or instillation of particulate materials in the lung have been shown to lead to an early and marked influx of Gr. Increased number of Le after asbestos exposure as a result of inflammatory response have been described by numerous authors (DZIEDZIC et al., 1993; HENDERSON et al., 1995; GREIM et al., 2001; MORIMOTO & TANAKA, 2001). In our study an increased level of Le was observed only 4 week after amosite exposure. Wollastonite exposure (4 weeks) resulted only in a tendency to increased number of Le. In the later phase (16 weeks) of exposure the total number of Le was not significantly changed for either fibre examined.

Lung exposure to noxious substances changes

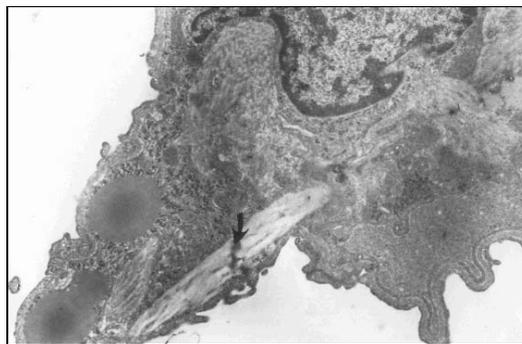


Fig. 4. Transmission electron microscopy of rat lung 16 weeks after treatment with a single intratracheal dose of 2 mg wollastonite per animal. A few collagen fibres accumulated subepithelially (arrow). Magnification: $\times 6600$.

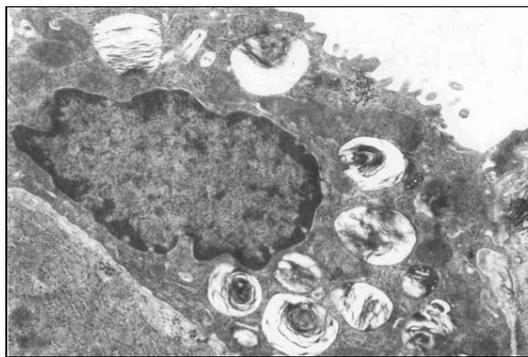


Fig. 5. Transmission electron microscopy of rat lung 16 weeks after treatment with a single intratracheal dose of 2 mg amosite per animal. An intermediate cells appeared in some of the alveoli with few microvilli. Magnification: $\times 8300$.

the differential cell count by increasing the proportion of inflammatory cells in particular that of Gr and Ly. However, a reduction in the absolute number of macrophages from the BAL fluid could also account for an increase in the proportion of inflammatory cells (DZIEDZIC et al., 1993; PITTET et al., 1997; HURBANKOVÁ & KAIGLOVÁ, 1999). In the present study we observed that % of AM was significantly decreased after both times of exposure to amosite (as compared with corresponding control groups). On the other hand % of Ly was significantly increased after both times (against the controls) and % of Gr was also increased but only after 16 weeks of amosite exposure. According to DZIEDZIC et al. (1993) and MORIMOTO & TANAKA (2001), long-term recruitment of Gr might be an important factor in prediction of lung metaplastic processes.

AM play a significant role in the mechanism, which regulates the response to fibrous dust exposure. Besides their phagocytic nature, AM are also important immuno-regulatory cells involved in defense mechanisms, as well as in the pathogenesis of numerous lung diseases. They cannot completely digest (phagocyte) long fibres – and come to “frustrated phagocytosis” and alveolar macrophages are activated. Activated AM release various cytokines, reactive oxygen intermediates and other mediators of the inflammatory reaction, that are important modulators of cell growth and cell differentiation (TARKOWSKI & GORSKI, 1991; DZIEDZIC et al., 1993; AFAQ et al., 1998; HURBÁNKOVÁ et al., 2001). Since AM are the first cells which come into contact with inhaled noxious substances, they constitute important and frequently utilized tools for examination of fibrous dust cytotoxicity. AM are the predominant cells present in BAL and changes in their number or function are factors in determining lung injury and characterizing the pathogenesis of such a response. Decreases in macrophage number, viability or phagocytic capacity may result in impaired clearance of inhaled materials (DZIEDZIC et al., 1993; TARKOWSKI & GORSKI, 1991; HURBÁNKOVÁ et al., 1998). Consequently, our observations of a significant decrease in % AM and viability (at 4 and 16 weeks) as well as significant depression of AM phagocytic activity (at 16 weeks) after amosite treatment indicate, as they do, a reduction in lung defence, constitute evidence of adverse effects of amosite fibres. After 16 weeks of wollastonite exposure, statistically significant decrease only in viability ($p < 0.05$) was occurred.

Changes in the type and quantity of proteins found in BAL fluid have been described in characterizing lung injury. Albumin is the most widely assayed protein in BAL fluid. It is primarily a serum protein and increased levels of this protein are generally due to endothelial damage and leakage of this protein across the endothelial and epithelial barriers. In general, increased levels of albumin in the lavage fluid indicate pulmonary edema, a common result produced by acute lung damage (DZIEDZIC et al., 1993; PITTEP et al., 1997). In the present study a statistically non-significant trend of increase of total amount of protein in BAL fluid was observed at either amosite exposure time.

A variety of enzymes washed from the lungs in the BAL fluid have been studied as markers of lung injury and disease. The presence of cytoplasmic enzymes in the extracellular supernatant of the BAL fluid is often used as a marker of cell

damage and cell lysis. In general, an increase in extracellular LDH is a good indication of acute cellular injury. As regards the levels of the enzymes examined in the present study (LDH, AcP, AP) significantly increased levels were observed only for LDH ($p < 0.01$) at both times after amosite (asbestos) treatment and after 16 weeks of wollastonite exposure ($p < 0.05$).

The pathogenicity of fibres depends apart from their size, also on other properties, e.g., concentration, physico-chemical properties, shape, surface area, charge and chemistry, contaminants adhered to their surface, long-term persistence in lung tissue, the way of splitting, etc. It is also related to the effective (retained) dose in the lungs, as well as their biological reactivity with lung tissue (CRAIGHEAD, 1993). In comparison with the wollastonite, asbestos fibres are much less soluble in the lung tissue and split longitudinally as a result of their crystalline structure, so that they length stays unaffected. Wollastonite fibres, in addition to being significantly more biosoluble than asbestos, split transversally, a property which enables their better elimination from the lungs (BELLMANN & MUHLE, 1992; WARHEIT, 1992). It is likely that these differences play a critical role in determining the higher toxicity of amosite asbestos indicated by our findings.

As far as the histological effects of the examined fibres are concerned, wollastonite was found to cause very minimal fibrosing alveolitis, with moderate progression by the 16th post exposure week (Fig. 1A,B). Fibrosis was degree I, according to Belt-King's classification (BELT & KING, 1945), by the 4th post-exposure week, and reached degree I-II by the 16th post-exposure week. In the work of TATRAI et al. (2004) the 6 month exposure to wollastonite did not induce significant fibrosis. These animals were instilled with lower dose – 1 mg per animal. In contrast, amosite gave rise to very definite, fibrosing chronic inflammation by the end of the 4-weeks (Belt-King II-III). After 16 weeks of exposure, progressive fibrosis with the obliteration of the lumina of bronchioli and alveoli (Belt-King III) could be detected (Fig. 2A,B).

Wollastonite did not lead to significant damage to the alveolar epithelium. In contrast, amosite caused injury of type I pneumocytes, as indicated by decreased RCA reactivity (Fig. 3A). This was followed by hypertrophy of type II cells, as indicated by extended MPA reactivity. Hypertrophic cells could be observed by transmission electron microscopy (TEM) 16 weeks of exposure (Fig. 3B). At this time, intermediate cells were seen, indicating the repair of type I cells. Subepithelial and

subendothelial accumulation of collagen was also observed at this time. This sequence of changes resulted in the degradation of the alveolo-capillary morphological unit (Figs. 4 and 5).

Type I pneumocytes are extremely sensitive to different noxious agents. The completeness of latter cells is a precondition for the integrity of the lungs owing to the inhibition of the clonal expansion of Ly (PAINE et al., 1991). Moreover, these cells normally release factors, such as prostaglandin E₂, which inhibit fibroblast proliferation and collagen production. Thus the damage, epithelial loss and incomplete repair can result in an imbalance of mediators and a shift towards fibroblast activation (HASCHEK & WITSCHI, 1979; CORRIN et al., 1985). This process impairs the appearance of cells, including macrophages, which are capable of producing different soluble mediators or cytokines – that trigger the proliferation of capillary endothelial cells, fibroblasts and smooth muscle cells (SHEPPARD & HARRISON, 1992). If the inflammatory reaction is prolonged, the tissue reconstruction often includes the proliferation of mesenchymal cells directing the outcome of process toward fibrosis (KOVÁCS & FRAZIER-JESSEN, 1994; KOVÁCS & DIPIETRO, 1994).

Our results suggested:

- the striking difference in the histological effects of wollastonite and amosite fibres, indicating a substantially higher lung toxicity of the latter, is in accordance with the corresponding effects observed on BAL markers of inflammation and cytotoxicity;
- wollastonite did not damage the alveolar epithelium whereas amosite caused the injury of type I pneumocytes (RCA reactivity decreased);
- only very moderate changes were found by histological examination and in BAL parameters after wollastonite exposure;
- the importance of *in vivo* testing of wollastonite as an asbestos substitute which is a very weak fibrogen;
- subsequent hypertrophy of type II cells in amosite treated lungs (MPA reactivity was extended and TEM showed hypertrophic cells after 16 weeks of exposure);
- the appearance of intermediate cells showing the repair of type I cells upon the effect of amosite (after 16 weeks of exposure). Consequent subepithelial and subendothelial accumulation of collagen after asbestos exposure.

Conclusions

In conclusion, wollastonite was found to cause very

moderate changes in BAL parameters of rats only 16 weeks after i.t. instillation with 2 mg of fibres. The effects observed on lung histology indicated minimal inflammation and fibrogenesis. In contrast, 2 mg of amosite fibres caused significant adverse changes in BAL parameters in both times of exposure, indicative of inflammation, accompanied by substantial lung injury which resulted in effective degradation of the alveolar-capillary unit. It is likely that this difference in the toxicity of the two fibres is at least partly related to the much shorter half time of elimination (low biopersistence) of wollastonite. The composition and structure of amosite may play role of its effects.

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