Physiol. Res. 46: 459 – 466, 1997

Some Bronchoalveolar Lavage Parameters and Leukocyte Cytokine Release in Response to Intratracheal Instillation of Short and Long Asbestos and Wollastonite Fibres in Rats

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Received January 20, 1997
Accepted July 14, 1997

Summary

We investigated the differences between the lavage parameters – including tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) release by lavage leukocytes – in control rats and in animals intratracheally instilled with short and long amosite and wollastonite fibres. These cytokines can play an important role in lung disease development after long-term exposure to some fibrous dusts. Short and long amosite and wollastonite fibres were intratracheally instilled in rats (1 mg/week) for ten weeks while saline was given to controls. To compare the harmful effects of these fibres, the number of leukocytes/ml of bronchoalveolar lavage (BAL), the number of alveolar macrophages (AM) per ml of BAL, AM:granulocyte (GR) ratios in lavage fluid, phagocytic activity and viability of AM, lactate dehydrogenase (LDH), acid phosphatase (AcP), and TNF-α and IFN-γ release by lavage leukocytes were investigated 3 months after the first intratracheal instillation. Compared with the controls, amosite short fibres significantly decreased the numbers of AM/ml BAL, and increased their phagocytic activity and AcP release. Long amosite fibres significantly decreased the numbers of AM/ml BAL, increased the number of granulocytes depressed the phagocytic activity and viability of AM, and significantly decreased the levels of TNF-α and IFN-γ in supernatants of cultured leukocytes. While wollastonite short and long fibre instillation did not significantly influence the parameters studied (except for a significantly increased number of leukocytes/ml BAL in wollastonite long fibres), amosite short and long fibres caused marked differences in these parameters, the long fibres being more effective.

Key words
Amosite – Wollastonite – Fibre size – Cytokines – Lavage parameters

Introduction

Murray (1900) was the first to publish a paper on necropsy findings in a worker occupationally exposed to asbestos. Subsequently, reports on the deleterious effects of asbestos fibres began to appear. After long-term exposure of men and experimental animals to asbestos, besides asbestoses and pleural hyalinoses neoplasms were also observed, e.g. lung carcinoma, pleural and peritoneal mesothelioma and mesotheliomas of other organs (Navrátil and Dobiáš 1973, Peto *et al.* 1982, Buchancová *et al.* 1988, Gombos *et al.* 1992, Morinaga 1993).

Mechanisms of the action of asbestos and other fibrous structures have not yet been fully explained. Therefore the relationship between tissue reaction and factors such as the duration of exposure, concentration, kinds, dimensions, surface properties of fibres, biopersistence, and individual disposition have been followed-up. Numerous papers suggest that the process of lung tissue injury after asbestos exposure depends on the dimension of fibres.

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 the disease. The most reactive are fibres, the diameter of which is less

than 0.25 μm and their length is greater than 8 μm (Stanton *et al.* 1981). Several studies also indicated that fibre types other than asbestos might be carcinogenic if their dimensions were similar (Tilkes *et al.* 1983, Pott *et al.* 1987).

There seems to be a direct proportionality between the size of a fibre and between its fibrogenic and carcinogenic effect, fibre diameter being probably more decisive than its length (Viallat *et al.* 1986). The pathogenicity of asbestos fibres also depends on the number and persistence of produced fibres.

Alveolar macrophages (AM) play a significant role in the mechanism regulating the response to fibrous dust exposure. Besides of being phagocytes, alveolar macrophages are also important immunoregulatory cells involved in defense mechanisms as well as in the pathogenesis of numerous lung diseases. They release various cytokines and other mediators of the inflammatory reaction that are important modulators of cell growth and differentiation. Macrophages may be additionally stimulated to release these factors by interferon-γ (IFN-γă originating from activated lymphocytes. Lymphocytes in turn remain under the influence of interleukin 1 (IL-1) released by macrophages. In the second stage, there is an increased accumulation of chemoattracted granulocytes which, when stimulated by the tumour necrosis factor-α (TNF-α), release increased amounts of radical oxygen intermediates. A highly increased level of radical oxygen intermediates, originating from macrophages and granulocytes, may cause lung tissue injury (Tarkowski and Gorski 1991).

We investigated the differences between the lavage parameters – the number of leukocytes/ml in bronchoalveolar lavage (BAL), the number of AM/ml BAL, AM:granulocyte (GR) ratios in lavage fluid, phagocytic activity and viability of AM, lactate dehydrogenase (LDH), acid phosphatase (AcP), TNF-α and IFN-γ release by lavaged leukocytes – in control rats and in animals intratracheally instilled with long and short amosite and wollastonite fibres each week (1 mg/week) for ten weeks. The above parameters were investigated 3 months after the first dust instillation.

Material and Methods

Female Wistar rats (Velaz Prague, Czech Republic, mean body weight 200 g) were used in our study. All animals were kept under non-infectious laboratory conditions (22±2 °C, 45 % relative air humidity, normal light-dark cycles) using an air-conditioning unit WOLF KG 100. Commercial diet (ST1) and tap water were available *ad libitum*.

The fibrous dusts studied were carefully administered intratracheally by a noninvasive procedure for ten weeks (the suspension – 1 mg fibres/0.1 ml saline solution per week) and a saline solution (0.1 ml per week) in the control groups. The exposed and control groups consisted of eight animals. Amphibole asbestos – amosite and wollastonite fibres belong to naturally occurring inorganic fibres. Wollastonite is used as a substitute of asbestos. The mean length, diameter, range and aspect ratio of these fibres is shown in Table 1.

Table 1. Mean length (L), diameter (D), range and aspect ratio (AR) of fibres

Fibres	L (μm)	Range (μm)	D (μm)	Range (μm)	AR
Amosite (short)	3.8	2.4–12.2	0.6	0.1–1.7	6.7
Amosite (long)	16.6	2.5–58.6	0.7	0.2–1.8	23.7
Wollastonite (short)	8.2	2.8–2.09	1.7	0.5–3.1	4.7
Wollastonite (long)	17.3	5.8–45.8	1.7	0.5–3.2	10.0

In the sample of amosite long fibres, 80 % of fibres were longer than 10–15 μm and in the sample of wollastonite long fibres, 75 % of fibres were longer than 10–15 μm.

Three months after the first exposure, the rats were anaesthetized by intraperitoneal Pentobarbital Spofa (50 mg i.p. per animal). Alveolar macrophages were harvested using a modified method according to 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/ml BAL-fluid was determined in a Bürker chamber (in 50 central squares x 5000). Differential cell counts were performed on May-Grünwald Giemsa stained preparations (the obtained BAL-fluid was centrifuged at 500 x *g* for 10 min at 4 °C). For the determination of phagocytic activity, LDH, AcP, TNF-α and IFN-γ levels, the cells were resuspended at a concentration of 10^6 AM/ml BAL-fluid. For the determination of LDH and AcP levels, the cell suspensions were centrifuged again at 8000 x *g* for 3 min. Lavaged leukocytes were cultured in suspension for 20 h in DMEM, and the supernatants were frozen at −20 °C until assay. The following parameters were investigated:

1. The cell number of leukocytes/ml BAL-fluid.
2. The number of AM/ml BAL-fluid.
3. Phagocytic activity of AM was investigated by the method of Fornusek *et al.* (1982) using 2-hydroxyethylmetacrylate particles (HEMA, Neosys, Prague). Fifty μl of HEMA particles in PBS (phosphate buffer) were added to 100 μl of BAL fluid and incubated for 60 min at 37 °C and shaken at short intervals. Staining was performed by the May-Grünwald-Giemsa method. Cells were considered positive when they phagocytized three or more particles.
4. Viability of AM – 200 μl of 0.25 % erythrosine solution were added to 200 μl aliquot of the cell suspension. The number of viable and non-viable cells was counted using a Bürker chamber.
5. The AM : GR ratios.
6. The levels of LDH were measured photometrically (Eppendorf, Germany) at wavelength 366 nm using LDH-UV kits Šarišské Michalany (Slovak Republic).
7. Measurement of acid phosphatase was carried out photometrically (by Specol Zeiss 1, Jena, Germany) at wave length 420 nm, using kits of Lachema Brno (Czech Republic).
8. TNF-α ELISA kit was purchased from GENZYME (Cambridge, MA). Lavage leukocytes were thawed at room temperature and added to microtitre wells precoated with anti-TNF-α monoclonal antibody. After incubation of the samples at 37 °C for 2 h and thorough washing of the wells, HRP-conjugated anti-TNF-α was added to the test wells. After one hour incubation at 37 °C, the excess of HRP-conjugated antibody was removed by washing. The substrate and stop solution were then added, and colour intensity was measured with ELISA reader (Dynatec) at 450 nm.
9. IFN-γ kit was obtained from GENZYME (Cambridge, MA). Thawed lavage leukocyte samples were added to microtiter wells precoated with monoclonal anti-IFN-γ antibody. Two hours after incubation at room temperature, goat anti-IFN-γ was added to the test wells. Another 2-hour incubation at room temperature with subsequent washing followed, then polyclonal donkey anti-goat-peroxidase was added. One hour after incubation at room temperature, the excess antibody was removed by washing. Substrate and stop solution were added and absorbance was read as described above.

All results were evaluated by Student's t-test.

Table 2. Some BAL parameters after 3 months' exposure to short and long amosite and wollastonite fibres

Parameters	Control	Amosite short	Amosite long	Wollastonite short	Wollastonite long
Number of leukocytes per ml BAL	327500	314375	488125*	400833	513750*
Number of alveolar macrophages/ml BAL	210833	141250**	108125***	192500	206875
Phagocytic activity of AM (%)	50.0	65.6***	40.1*	57.2	42.1
Viability of AM (%)	89.0	88.0	84.5**	87.0	88.0
AM:GR ratios (% GR)	0.33	0.50	2.25*	0.83	1.13

** $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

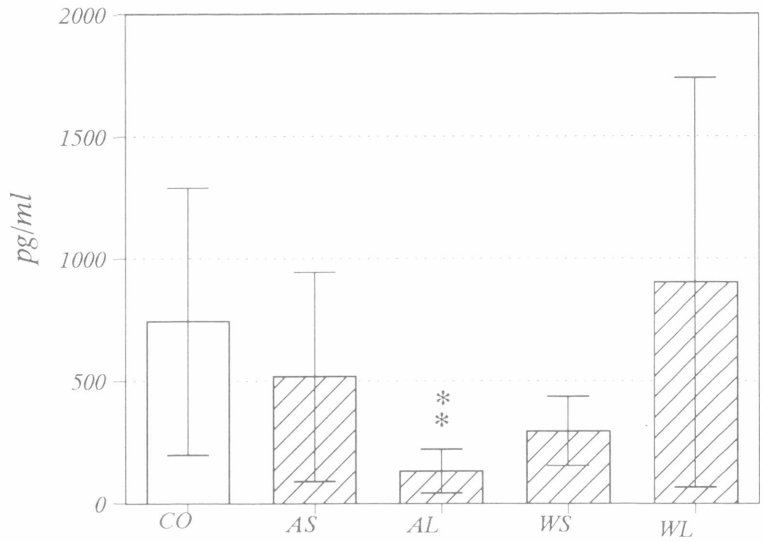

Fig. 1. *The level of IFN-γ in the BAL-fluid after exposure to short and long amosite and wollastonite fibres – three months after the first instillation.*

CO - Control AS - Amosite short fibers WS - Wollastonite short fibers
AL - Amosite long fibers WL - Wollastonite long fibers

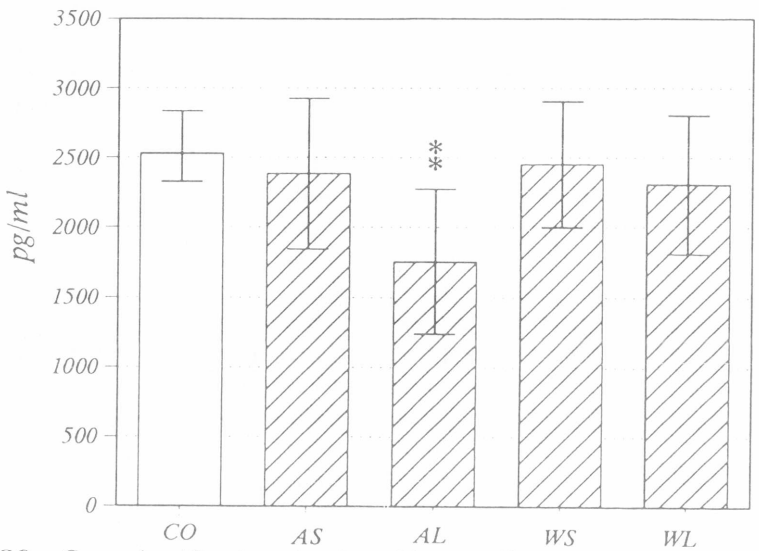

Fig. 2. *The level of TNF-α in the BAL-fluid after exposure to short and long amosite and wollastonite fibres – three months after the first instillation.*

CO - Control AS - Amosite short fibers WS - Wollastonite short fibers
AL - Amosite long fibers WL - Wollastonite long fibers

Results

Three months after the first instillation, the number of rat leukocytes in ml of BAL-fluid was significantly increased in the groups treated with long amosite ($p<0.05$) and wollastonite fibres ($p<0.05$), as compared with the control group. Exposure to short amosite and wollastonite fibres did not significantly affect the number of leukocytes at this time period (Table 2).

In comparison with the control group the number of AM in BAL-fluid was significantly decreased after exposure to either short ($p<0.01$) or long ($p<0.001$) amosite fibres. Exposure to both short and long wollastonite fibres did not significantly affect the number of AM in the BAL-fluid. As compared with the control group, phagocytic activity of AM was significantly increased in group treated with short amosite fibres ($p<0.001$), while it was decreased after exposure to long amosite fibres ($p<0.05$). The viability of AM was significantly decreased after exposure to long amosite fibres only. Treatment with other types of fibrous dust did not affect the viability of AM at this time point.

A comparison of the percentage of granulocytes (i.e. AM:GR ratio) in control and treated groups documents that this parameter was significantly increased after treatment with long amosite fibres ($p<0.05$), while other fibres did not exert any appreciable influence on this parameter (Table 2).

The level of IFN-γ released into the BAL-fluid was significantly decreased only after exposure to long

amosite fibres ($p<0.01$), while other tested fibrous dusts did not affect the release of IFN-γ into the BAL-fluid significantly (Fig. 1).

The level of TNF-α derived from AM was significantly decreased after exposure to long amosite fibres as compared with control groups. Other tested fibrous materials did not cause any changes in the release of TNF-α at this time point (Fig. 2).

The level of acid phosphatase was significantly increased only in the group exposed to short amosite fibres ($p<0.01$) while that of LDH was not affected by any of the tested materials.

Discussion

The processes which lead to morphological changes of the lungs after long-term exposure to some industrial mineral fibrous dusts are still not clear, they involve many molecular and cellular reactions, including an immunological response. The important role which macrophages fulfil in the immune system and in asbestos-related diseases is unquestionable. The body of evidence, which has been accumulating during the last decades, indicates that the macrophage role extends far beyond phagocytosis. Besides their immunoregulatory and secretory function in defense mechanism, they may control the activity of other cells, such as neutrophils, lymphocytes and fibroblasts. The effect of mineral fibres on AM may injure the plasmatic membrane and lead to the release of lysosomal and cytoplasmic enzymes and the impairment of surrounding tissues as well as generation of reactive oxygen radicals, which are involved in impairment of lung alveolar epithelium and play an important role in the development of the inflammation reaction. Moreover, asbestos fibres stimulate inflammatory cells to secrete various cytokines, growth factors and proinflammatory mediators. The interaction between mediators released from macrophages or other inflammatory cells and the target cell population has been postulated to initiate a sequence of events culminating in fibrosis of the lungs and pleura, bronchogenic carcinoma and malignant mesothelioma (Nouza and John 1987, Ferenčík 1989, Tarkowski and Gorski 1991, Brain 1992, Brody 1993, Hurbánková and Ulrich 1992, Hurbánková and Kaiglová 1993, Hurbánková 1994).

Fibre length has been found to be the major descriptor of the ability of industrial fibres of different types to cause lung pathology (Davis *et al.* 1986). Stanton's and Pott's hypothesis regarding the pathogenicity of asbestos was based on experiments with precisely defined geometrical properties of fibres. They found that the pathogenicity of fibres tended to increase with diameter less than 0.25 μm and length greater than 8 μm (Stanton *et al.* 1981, Mohr *et al.* 1984, Pott *et al.* 1987). Based on animal experiments with asbestos, erionite and MMMF also Jaurand (1989) suggested that the size of fibres is an important factor of carcinogenicity. Nevertheless, the type of fibres and their physico-chemical properties also play an important role. Consequently, besides the geometry and number of fibres the physico-chemical properties as surface chemistry, chemical composition and surface area play a significant role which may be included in the term biopersistence (McClellan and Hesterberg 1994).

At present, the question of the toxicity of asbestos substitutes is an area of extensive research. However, there is only limited knowledge concerning the biological effects of these fibres.

Our study, designed on the basis of these experiments, was aimed to investigate the possibility that the different influence of particular fibres on some parameters of AM and other leukocytes in BAL-fluid is associated with the length of the fibres. In this study we compared the biological effects of long and short fibres of amosite-naturally occurring silicate of the amphibole asbestos group, to those of wollastonite, naturally occurring calcium silicate used as a substitute of asbestos in some applications. Wollastonite fibres are rather similar in form, length and diameter to amphibole asbestos fibres, but they are different mineralogically.

The results of our experiments showed that the number of leukocytes/ml BAL-fluid was significantly increased as compared to the controls, but only in groups exposed to long asbestos and wollastonite fibres. The number of AM/ml BAL-fluid was decreased in the groups exposed to both short and long amosite fibres, the effect of long amosite fibres being more pronounced ($p<0.001$). They also significantly decreased the phagocytic activity and viability of AM. The decrease in macrophage number or phagocytic capacity may result in a reduced clearance of inhaled materials and thus to increase the effective dose of the potentially injurious agent (Dziedzic *et al.* 1993). It is possible that this decrease in the number of recovered AM is due to the toxicity of particles acting on the stem cell population. Normally, AM are maintained in a steady-state either by self-renewal or by clonal growth of a putative stem cell: alveolar colony forming cells (AL-CFC) (Oghiso *et al.* 1992). Hence, a decrease of the AM number/ml BAL in both amosite exposed groups studied may have been caused by the toxicity of amosite fibres on the stem cell population. The results of Donaldson *et al.* (1988) indicate that after exposure to chrysotile macrophages were not available for lavage towards the end of the exposure due to their potential deposition in granulation tissue. Development of granulomas consisting of lymphocytes, fibrocytes and collagen fibres with multinucleic cells around fibres after exposure to long amosite fibres was also confirmed by histological observations, while no histological changes were observed at this time interval in the groups

exposed to other fibrous materials. This fact might explain a restricted availability of macrophages in BAL-fluid after exposure to long amosite fibres.

Granulocytes represent an important part of the acute response of fibrous dust exposure in animal models (Dodson and Ford 1991). Numerous papers reported increased number of neutrophils in BAL-fluid after exposure to asbestos and other fibrous dusts in the acute phase (Mossman *et al.*, 1991, Rom 1991, Petruska *et al.* 1991). In our study, a significant increase of granulocytes was recorded only in the group exposed to long amosite fibres ($p<0.05$). These fibres can persist as extracellular fibres in the tissue due to their large size, and thus can be the cause of the inflammatory response.

According to hypothesis, the development of pathological processes caused by exposure to asbestos and other fibrous materials involves the action of various cytokines. The most important ones implicated in these processes are IL-1, TNF-α, IFN-γ (Carre and Leophonte 1993, Schwartz *et al.* 1993, Zhang *et al.* 1993). This prompted us to investigate the levels of IFN-γ and TNF-α after exposure to the studied fibrous dust. The results of these experiments are surprising, since the levels of the mentioned cytokines in lavage leukocyte cultures were significantly decreased after exposure to long amosite fibres.

IFN-γ is released by T-lymphocytes and by NK-cells and plays an important role in regulation of immune reactions . One of its major properties is its ability to suppress growth of both normal and tumour cells. IFN-γ acts as an activator of macrophages, which is in good agreement with our results showing that the decreased levels of IFN-γ after exposure to long amosite fibres are accompanied by decreased number, phagocytic activity and viability of AM.

Normally, the processes of inflammation and response to injury are associated with augmented release of TNF-α (Larrick and Kunkel 1988). Interestingly, our results showed that the deposition of long amosite fibres in the airway space significantly decreased the release of TNF-α by AM. Li *et al.* (1992) reported that, following intratracheal instillation of 5 mg of crocidolite asbestos, the pleural leukocytes secreted less TNF-α than the control cells. They assumed that this might be caused either by a blockade at the level of TNF-α gene expression or at the level of its release, which would explain the prevalence of TNF-α-inhibiting activity in the culture. Similar results were also described by Quellet *et al.* (1993), who found significantly decreased levels of TNF-α in rats exposed to UICC chrysotile for one or three weeks. However, higher levels of TNF-α production were recorded in this group after 6 weeks of exposure. Their data indicate that lower levels of TNF-α resulted from inhibition at the gene expression level and provide evidence for bidirectional modulation of TNF-α production by AM during inflammatory reactions. However, neither of these papers took into consideration the size of fibrous material to which the animals were exposed. We suppose that the differences in cytokine levels after exposure to fibrous dusts may result from differences in dust size, duration of exposure and fibre dose. In our study, the doses of fibrous dusts were higher than the potential inhalatory exposures in humans because, when investigating the differences in their mechanism of action, we intended to reach the most conspicuous changes in the studied parameters.

The results of our study indicate that long amosite fibres may contribute to the immunosuppression in an exposed host by affecting the production and activity of the immunoregulatory cytokines IFN-γ and TNF-α and thus facilitate the development of the disease. In contrast, the exposure to short amosite fibres did not suppress any of the studied parameters, with the exception of the decreased number of AM/ml BAL-fluid. A significant increase of phagocytic activity and acid phosphatase levels observed in this group of animals would indicate that a persistently operating defense mechanisms is involved. Exposure to either short asbestos fibres or short and long wollastonite fibres did not have any adverse effect at this studied time interval. Thus, the results of our study confirmed the hypothesis that, as far as asbestos is concerned, its bioreactivity depends on the length of this fibres. However, this did not apply (at least in the experimental model which we used) in the case of wollastonite. The results of our study indicate that mechanism of action of these two types of fibres is different and this may be due to differences in their biopersistence.

Acknowledgements

The authors thank the company NAFTA a.s. Gbely for providing the financial support for purchasing kits for the cytokine evaluation and Anna Francová and Helena Turazová for excellent technical assistance.

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