Characteristics of alveolar cells and soluble components in bronchoalveolar lavage fluid from non-smoking aluminium potroom workers

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ABSTRACT Aluminium potroom workers have been reported to develop severe pneumoconiosis and bronchial hyperreactivity. The influence of inhalation of aluminium oxide and fluorides on the alveolar milieu was studied by bronchoalveolar lavage (BAL) in 14 male non-smoking potroom workers; 28 non-smoking healthy volunteers served as controls. The total numbers, concentrations, and proportions of various alveolar cells did not differ between the groups. The concentrations of albumin and fibronectin in BAL fluid were significantly higher ($p < 0.01$ for both) in the exposed workers, reflecting an increased alveolar capillary permeability and an activation of alveolar macrophages (AMs). The concentration of angiotensin converting enzyme, another AM marker, was, however, decreased ($p < 0.01$) in the workers. The concentration of hyaluronan, a fibroblast marker, did not differ between the groups. AMs from workers had a decreased capacity ($p < 0.05$) to interact with yeast C3b particles but not to ingest them. The expression of HLA-DR and OKM1 on the cell surfaces of AMs were equal in the two groups. The BAL findings were not accompanied by restrictive lung disease in the workers. The fact that only a discrete alveolitis was found in the potroom workers may be due to a low grade of exposure to alumina and fluorides and to frequent use of respiratory protection equipment.

Aluminium potroom workers are exposed to alumina (aluminium oxide) dust and to particulate and gaseous fluorides. Aluminium plant employees may develop severe pneumoconiosis¹⁻³ and bronchial hyperreactivity.⁴⁻⁸ No relation between atopic constitution and the development of airways obstruction in potroom workers has been observed.⁹

During the past decade bronchoalveolar lavage (BAL) has made possible the study of the alveolar milieu with regard to cells and soluble components under the influence of various agents. For example, the effect of smoking on cells and non-cellular substances has been well documented in several studies.¹⁰ The BAL technique also offers the opportunity to study the effects of occupational exposure to various agents potentially harmful on the alveolar space; those exposed to alumina have not to our knowledge been investigated.

To analyse the influence of the inhalation of alumina dust and fluorides on alveolar cells and soluble components we performed BAL in non-smoking male potroom workers. The alveolar cell population was characterised with regard to cell surface markers and phagocytic activity with flow cytofluorometry. The activity of the cells and the permeability of the alveolar capillary membrane were studied by measuring the concentration of fibronectin, hyaluronan, angiotensin converting enzyme (ACE), and albumin.

Materials and methods

STUDY POPULATION

Fourteen healthy male volunteers (mean age 42, range 24-62) working in the potroom of the aluminium production plant GA-Metall in Sundsvall, Sweden, were included in the study. The mean duration of employment was 12.9 ± 9.0 years. Exposure assess-

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ments for different working tasks have been performed by measurements on eight hour samples from eight workers with the same type of occupation. The mean values of exposure of the study population were 1.77 mg/m³ (range 0.49–4.50) for total dust and 0.31 mg/m³ (range 0.10–0.50) for gaseous and particulate fluorides. More than 65% of the alumina particles were smaller than 6.3 μ . None of the workers had smoked during the past two years; previous cigarette consumption was 4.3 ± 8.2 (mean $\pm$ SD) pack-years. All had a normal chest x ray film and ECG.

Standard spirometry was performed in a rolling seal spirometer and lung volumes were measured in a body box. Diffusion capacity was measured by the single breath carbon monoxide technique. A group of 20 office workers from the same factory served as controls.

The control group for the BAL data consisted of 28 non-smoking healthy volunteers (mean age 25, range 19–31) with normal chest x ray films. The study had the approval of the local ethics committee and all subjects gave informed consent.

BRONCHOALVEOLAR LAVAGE

After premedication with morphine and scopolamine and local anaesthesia with 2% lignocaine (Xylocain, Astra, Södertälje, Sweden) a flexible fiberoptic bronchoscope (BF Type 4B2, Olympus Optical Co Ltd, Japan) was wedged in a bronchus in the middle lobe. Sterile saline solution at 37°C was instilled in five aliquots of 50 ml and gently suctioned back as described earlier.¹¹ The fluid was collected in a plastic bottle kept on ice.

HANDLING OF ALVEOLAR CELLS

The BAL fluid was strained through a double layer of Dacron nets. After centrifugation at 400 g for five minutes at +4°C the supernatant was poured off and stored at –70°C until analysed further. The cells were resuspended in Hank's balanced salt solution and a total cell count was performed in a Bürker chamber. The viability was evaluated by means of excluding trypan blue. The cell suspension was centrifuged a second time at 400 g for five minutes and the cells were resuspended in 4 ml of RPMI 1640 culture medium (Labasco, Uppsala, Sweden) with the addition of gentamycin 5000 μ g/ml. The cell suspension was kept on ice and transported to the laboratory the same day.

A differential cell count was performed after the preparation of cytocentrifugal smears in a Cytospin 2 apparatus (Shandon, England) at 500 rev/min for three minutes. Cells were stained according to May-Grünwald Giemsa and 500 cells were counted.

BIOCHEMICAL ANALYSES IN BAL FLUID

Albumin (L-Alb) was determined by rocket

immuno-electrophoresis¹² and expressed as mg/l.

Angiotensin converting enzyme (L-ACE) activity was analysed according to Lieberman¹³ in concentrated BAL fluid as earlier described¹¹ and expressed in U/L (nmol hippuric acid/min/l).

Fibronectin (L-Fn) was assayed by a double sandwich ELISA developed in our laboratory. The aliquots of BAL fluid and EDTA plasma were defrozen at 37°C. Briefly, microtitre plates (Nunc, Denmark) were coated with rabbit antihuman fibronectin antibodies (Dakopatts AS, Glostrup, Denmark) in phosphate saline buffer, pH 7.2. After the addition of unconcentrated BAL fluid samples the plates were incubated at room temperature for two hours. Horse radish peroxidase labelled antihuman fibronectin (Dakopatts AS, Glostrup, Denmark) was added as second antibody and the plates were incubated for one hour. The amount of bound peroxidase, which is proportional to the amount of fibronectin in the sample, was measured by analysing its enzymic activity on 100 μ l orthophenylendiamine. The reaction was blocked by the addition of 150 μ l 1 M H₂SO₄ after incubation for six minutes and the absorbance at 492 nm was registered. Serum fibronectin of nephelometric quality from Behring-Hoechst (Frankfurt am Main, GFR) was used as a standard. Concentrations of L-Fn were expressed in μ g/l. The detection limit was 10 μ g/l. Intra-assay and interassay CVs were 3.7% and 6.4%, respectively.

Hyaluronan (L-HA) was analysed in principle according to Engström-Laurent using the Pharmacia HA test kit.¹⁴ Concentrations were expressed in μ g/l.

PHAGOCYTIC ASSAY

A phagocytic assay using fluorescence quenching to differentiate between attachment and ingestion and recently adapted for flow cytometry was used to study receptor mediated phagocytosis.¹⁵ Cell pellets of alveolar macrophages (AMs) containing $3\text{--}6 \times 10^5$ cells were resuspended in 200 μ l of Geys buffer (supplemented with Ca²⁺ and Mg²⁺) containing 5×10^6 serum opsonised heat killed yeast particles (yeast-C3b) per millilitre as prey. After incubation at +37°C for 15 minutes the reaction was interrupted by adding ice cold buffer. Fluorescence of attached particles was quenched by adding trypan blue. The cell samples, treated and not treated with trypan blue, were then examined in an Ortho Spectrum III flow cytofluorometer (Ortho Diagnostic Syst, Westwood, Ma, USA). AMs interacting with fluorescent particles, attached or ingested, were analysed in samples not treated with trypan blue. This was expressed as “% interacting cells.” The percentage of AMs with ingested particles (“% ingesting AMs”) was obtained by dividing the percentage of fluorescent cells after addition of trypan blue with the percentage of fluorescent cells before addition of the dye. This

measurement was regarded as the ability of AMs to ingest particles.

ANALYSIS OF CELL SURFACE MARKERS

Before AMs were used in the immunoassays for surface antigens the autofluorescence was quenched with a technique that has recently been developed in our laboratory. Briefly, 100 μ l of room tempered n-octyl-beta-D-glucopyranoside (0.1% in PBS without EDTA) was added to the cell pellet. The suspension was mixed and incubated for five minutes in room temperature. Then 100 μ l of crystal violet (2 mg/ml in sodium chloride) was added before a second incubation for five minutes on ice. The cell suspensions were washed twice in cold PBS-EDTA. The cell pellets were resuspended in 100 μ l of PBS-EDTA and were then used in the immunoassays for surface antigens. Ten microlitres of FITC conjugated monoclonal antibodies against HLA-DR (Becton-Dickinson Immunocytometry Systems, Mountain View, Ca, USA) or non-conjugated OKM1 (Ortho Diagnostic Systems Inc, Raritan, NJ, USA), diluted 1/10 in PBS-EDTA, were added to the cell suspensions and incubated on ice for 30 minutes. Tubes containing anti-HLA-DR were washed and examined as described below. Tubes containing OKM1 were resuspended and incubated on ice with 100 μ l of FITC conjugated rabbit-F(ab')₂ -antimouse immunoglobulin (Dakopatts AS, Glostrup, Denmark), diluted 1/20 in PBS-EDTA, for another 30 minutes. The cells were finally washed twice, resuspended in 1 ml cold PBS-EDTA, and examined by flow cytometry. The expression of HLA-DR antigens and C3bi-receptors (OKM1) was measured as the mean fluorescence intensity (MFI) of the cell population after subtraction of the background level calculated from control samples.

Statistical analysis

The observed values were not normally distributed and the results are given as medians and interquartile ranges unless otherwise stated. Results were compared with the Mann-Whitney test and correlations calculated according to Spearman.

Results

LUNG FUNCTION

The potroom workers showed moderate obstructive changes compared with controls. Mean values ($\pm$ SD) of vital capacity, total lung volume, residual volume (RV), forced expiratory volume in one second (FEV₁), and diffusion capacity for carbon monoxide, in percent of predicted values, were $95.1 \pm 8.5\%$, $101.1 \pm 10.4\%$, $126.8 \pm 28.8\%$, $94.3 \pm 9.2\%$, and $89.6 \pm 13.3\%$, respectively. RV and FEV₁ differed significantly from controls ($p < 0.01$, $p = 0.05$) but the other values did not differ between the groups.

GENERAL LAVAGE DATA

As shown in table 1 in the potroom workers the median recovery of the instilled fluid was 66%; 75% of the cells were viable. The total cell numbers, the cell concentrations, and the proportions of the various types of cells from workers and controls did not differ significantly.

SOLUBLE BAL COMPONENTS

In the cell free BAL supernatant there was a significantly higher ($p < 0.01$) concentration of albumin in workers compared with controls, medians 61 mg/l and 31 mg/l respectively (figure). Moreover, the fibronectin concentration in workers (median 75 μ g/l) was raised ($p < 0.01$) compared with controls (20 μ g/l). The ACE concentration (median 201 U/l) was significantly decreased ($p < 0.001$) in the workers compared with controls (340 U/l). The hyaluronan concentration did not differ significantly between the two groups, the medians being 22 μ g/l and 26 μ g/l respectively.

There was a significant correlation ($r = 0.56$, $p < 0.05$) between the concentrations of albumin and fibronectin in the BAL fluid from potroom workers. No relation was found between any other of the various soluble components nor between any of these and the total alveolar cell number in the workers.

PHAGOCYTIC FUNCTION AND SURFACE ANTIGEN EXPRESSION OF AMS

The percentage of AMs interacting with yeast C3bi particles showed a small but significant ($p < 0.05$)

Table 1 General characteristics of the BAL fluid recruited from 14 non-smoking aluminium potroom workers and 28 healthy non-smoking volunteers (controls). Data are given as medians with interquartile ranges

Group	Recovery (%)	Viability (%)	Total cell count ($\times 10^6$)	Cell conc ($\times 10^6$ /l)	Differential cell count		
					AM	Ly	PMN
Potroom workers	66 (57-73)	75 (65-78)	9.9 (6.4-16.0)	69.1 (45.7-84.2)	87.5 (85.0-95.0)	10.0 (5.0-13.0)	1.0 (0.0-2.0)
Controls	68 (64.8-75.2)	84 (80.5-87.0)	14.5 (11.7-21.6)	94.0 (65.0-121.5)	88.7 (86.2-93.4)	9.0 (5.5-11.2)	1.2 (0.6-2.3)

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Results of biochemical analysis of non-cellular components in BAL fluid from 28 controls and 14 potroom workers. Boxes cover values within interquartile ranges, central horizontal line is at median, and vertical lines indicate ranges.

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

Table 2 Expressions of HLA-DR antigens and OKM1 (C3bi receptors) on alveolar macrophages (AM) from aluminium potroom workers and healthy controls. Expressions are given as mean fluorescence intensity (MFI) of the AM population as measured in a flow cytometer immunoassay

	HLA-DR		OKM1 (C3bi)	
	Median	Range	Median	Range
Workers (n = 11)	225	154-233	68	54-107
Controls (n = 13)	203	198-212	85	51-105

decrease in workers (median 40%, interquartile (iq) range 36-44) compared with controls (median 47%, iq range 41-49). The percentage of ingesting AMs did not differ between workers (median 63%, iq range 54-67) and controls (median 67%, iq range 60-72) implying that the ability of the AMs to ingest yeast C3b was not altered in the workers.

No significant differences in expression of HLA-DR and OKM1 (C3bi-receptors) could be detected between workers and controls as measured by the immunoassay in flow cytofluorometry (table 2).

Discussion

In the present study the influence of occupational exposure to alumina dust and particulate and gaseous fluorides on the alveolar milieu was analysed. Since others have found that potroom workers are at risk of developing pulmonary fibrosis¹⁻³ we expected to find signs of a high intensity alveolitis. The alveolar cell concentration and the distribution of the cell sub-populations in the potroom workers, however, did not

differ from the controls. The lack of increased recruitment of cells to the alveolar space in these workers indicate a low grade generation of chemotactic factors. The alveolar cells recruited from workers showed only a small decrease in receptor mediated phagocytosis and no effect on the phagocytes' ability to ingest particles. Furthermore, the expression of the DR-antigen and receptors for complement factor C3bi (OKM1) did not differ in workers and controls.

The analysis of non-cellular components in the BAL fluid offers further means to evaluate the presence and intensity of alveolitis.¹⁰ In the present study an increased concentration of albumin was found in the lavage fluid. Albumin leakage to the alveolar space is a sensitive marker of a local inflammatory reaction.¹¹ Despite this indication of a local inflammation analysis of fluid phase markers of macrophage activation showed conflicting results since fibronectin was significantly increased but ACE significantly decreased in workers. The correlation ($r = 0.56$, $p < 0.05$) found between the lavage albumin and fibronectin concentrations may possibly indicate a concomitant leakage of the two substances into the alveolar space. Fibronectin found in the lavage fluid could partly originate from cells outside the alveoli. The discrepancy in the findings of raised fibronectin and lowered ACE concentration in the lavage fluid could alternatively be explained by different regulatory systems for synthesis and release of the two substances from the AMs. Both substances may play a part in the inflammatory process eventually leading to fibrosis. Thus fibronectin plays a part in attracting fibroblasts and attaching cells to the intercellular matrix¹⁶ and ACE may interact in the inflammatory process. The low intensity alveolitis in these potroom workers did not result in restrictive lung disease and the normal lavage concentration of hyaluronan, a

marker of fibroblast activation, is in accordance with this finding.¹⁷

Exposure to alumina and fluorides was well below the Swedish threshold limit values and the inhaled dose was also reduced by the frequent use of respiratory protection equipment in this plant. This might explain why the intensity of the alveolitis is of low grade.

In conclusion, in the present study analysis of BAL fluid recruited from aluminium potroom workers shows a low grade alveolitis expressed as an increased alveolar capillary permeability and altered macrophage function. It is not known if such a discrete alveolitis may play a part in the development of fibrosis. Most of the knowledge about cellular and fluid phase markers of inflammation in the alveolar space is based on reactions with more inflammatory substances than alumina. Also, it is often based on analysis of individuals with clinically manifest diseases. Whether the low intensity of the alveolitis found in the aluminium potroom workers in this study depends mainly on qualitative or quantitative factors could not be clarified but will be further analysed in continuing studies.

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