Iron content in human alveolar macrophages


ABSTRACT: Intracellular iron can be estimated semi-quantitatively by histochemical determination using the ferrocyanide reagent’s score. Particle-induced X-ray emission (PIXE) allows accurate determination of various elements including iron in cells and biological fluids. Both techniques have been used to measure iron in alveolar macrophages gathered by bronchoalveolar lavage. The purpose of this study was to investigate the clinical usefulness of the PIXE technique in occupational respiratory medicine and in various pulmonary diseases.

Using the PIXE method, we measured the iron content of alveolar macrophages in healthy subjects, with and without occupational exposure to iron dust, and in patients with pulmonary diseases (chronic obstructive pulmonary disease (COPD), lung cancer, Goodpasture's syndrome). Our results were then compared with those obtained with the ferrocyanide reagent.

Intramacrophagic iron was 0.33±0.21 μg·10^-5 (mean±SD) cells in healthy non-smoking subjects without occupational exposure. Intramacrophagic iron was increased in smokers, iron-steelworkers, and in patients with COPD or lung cancer even in the absence of pulmonary haemorrhage. The two patients with Goodpasture's syndrome had high intramacrophagic iron content. About 80% of the whole bronchoalveolar lavage fluid iron content was in the cells. Mean iron content of blood monocytes, lymphocytes and neutrophils of eight healthy subjects was significantly lower than that of alveolar macrophages. A significant correlation was found between iron determination by the PIXE method and the ferrocyanide reagent’s score (r=0.89).

We conclude that intramacrophagic iron may be increased in steelworkers and subjects with pulmonary haemorrhage, but also in asymptomatic smokers, in COPD and lung cancer patients without occupational exposure to iron dust.

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Alveolar macrophages, the predominant cells normally found in bronchoalveolar lavage fluid (BALF), have many important functions including phagocytosis, antimicrobial activity and the processing of antigens in pulmonary immune reactions. Iron, the most abundant metal in the human body, is an essential element of many proteins, playing a key role in oxygen transport and utilization. Alveolar macrophages bear transferrin receptors on their surface and have high intracellular ferritin content [1]. They may process haemoglobin from ingested red cells to form ferritin and haemosiderin [2].

Intracellular iron can be easily shown by the ferrocyanide reagent [3-5]. Finlay and co-workers [6, 7] applied this technique to semi-quantitatively measure iron in alveolar macrophages of subjects suffering from occult intrapulmonary haemorrhage and proposed a score based on the intensity of the staining. This technique has never been validated using a more quantitative method.

Iron can be accurately measured by the particle-induced X-ray emission (PIXE) method and this technique has been applied to measure iron content in BALF [8]. The aim of this study was to investigate the clinical usefulness of the PIXE method in the determination of iron content in occupational respiratory medicine and in various pulmonary diseases (including occult pulmonary haemorrhage). We therefore used this method to measure iron content of alveolar macrophages in healthy subjects, with and without occupational exposure to iron dust, and in patients suffering from various pulmonary diseases. We also compared these results with those obtained with the ferrocyanide reagent’s score.

Subjects and methods

Subjects

One hundred and ninety three subjects, including healthy volunteers and in- or out-patients suffering from respiratory diseases, without history of pulmonary haemorrhage (with the exception of one subject with Goodpasture’s syndrome), were studied and divided into two groups:
Group 1. Subjects with no exposure to industrial dust:
  i) 43 healthy volunteers (no respiratory symptoms and normal chest X-ray), aged 47±14.5 yrs mean±sd; 19 nonsmokers and 24 smokers;
  ii) 44 patients suffering from lung carcinoma, aged 62±10.3 yrs; 17 nonsmokers (including 11 ex-smokers) and 27 smokers;
  iii) 6 smokers suffering from chronic obstructive pulmonary disease (COPD), aged 61±16 yrs;
  iv) 2 subjects (58 and 47 yrs) with Goodpasture's syndrome (1 smoker).

Group 2. Subjects with exposure to industrial dust:
  i) 61 blast-furnace workers, aged 57±5.3 yrs, 24 nonsmokers (including 6 ex-smokers) and 37 smokers: 41 healthy volunteers, 9 COPD, 6 asbestosis, 4 lung carcinoma and 1 siderosis;
  ii) 29 coke-oven workers, aged 54±6.6 yrs, 20 smokers and 9 nonsmokers (including 3 ex-smokers): 19 healthy volunteers, 6 COPD, 3 lung carcinoma and 1 asbestosis;
  iii) 8 welders, aged 56±16 yrs, 6 smokers and 2 nonsmokers: 4 lung carcinoma, 1 siderosis, 3 healthy subjects.

Bronchoalveolar lavage

All of the subjects underwent a bronchoalveolar lavage during fibroptic bronchoscopy which was performed under local anesthesia according to a standardized technique [9, 10]. Sterile saline (200-250 ml) at 37°C was injected in 50 ml boluses into a subsegmental area of the middle lobe. The BALF was recovered by mild aspiration. BALF yield was 50-90% of the volume injected and was immediately processed. BALF was performed in the healthy volunteers with the approval of the Ethics Committee of the Centre Hospitalier Universitaire de Liège.

BALF analysis

BALF samples were centrifuged (800 xg, 10 min) and pelleted cells resuspended in 1 ml of saline solution. Cells were counted in a Thoma cell. A cell smear was stained with Diff-Quick reagent (Harleco) and a differential count was performed by examining 300 cells.

PIXE method (11)

In each subject, a cell suspension containing $1\times10^6$ washed alveolar cells was deep frozen and lyophilized for 24 h. The residue was suspended in 250 µl of Yttrium solution (internal standard: 400 µg Y·ml$^{-1}$) using a Paramix II mixer. Thin targets were prepared by pipetting 25 µl aliquot of the resulting suspension and 25 µl of a 10% polyvinylpyrrolidone (PVP) solution onto a 3 µm thick polypropylene film, stretched on a commercial 24×36 mm slide frame. This type of backing was found to be free from any serious contamination. The PVP was used to avoid local crystallization of the sample. The droplet was then air-dried at room temperature.

A beam of 2.5 MeV protons, originated by the variable energy cyclotron of the "Centre de Recherche du Cyclotron", University of Liège, was used to bombard the sample under vacuum. The beam intensity was held up to 50 nAmp to avoid damaging the sample. The characteristic X-rays produced in the sample were detected by use of a Si(Li) detector. The resulting spectra obtained by performing an analogic-digital conversion of the electrical pulses emerging from the amplifier was used to measure the area of the different x-ray lines. These peaks were processed by means of a computer program in order to subtract the inherent background mainly due to the "bremstrahlung" of the secondary electron produced in the sample itself. Quantitative analysis was performed using the internal monitor method. A calibration curve was established by bombarding standard targets containing precise quantities of elements. This curve (Az) gives for each atomic number:

$Az = \frac{Sz \cdot my}{Sy \cdot mz}$

where $Sz$=peak area of the $z$ element; $Sy$=peak area of the internal standard; $mz$=mass of the $z$ element (µg); $my$=mass of the internal standard (µg).

The concentration $C$ in the $z$ element was obtained as follows:

$Cz = \frac{Sz \cdot my}{Sy \cdot Az \cdot M}$

where $Sz$, $Sy$ and $my$ correspond to the bombardment of the unknown sample. $M$=the total mass (or the total number of cells) of the unknown sample. Results were expressed in µg·10$^6$ cells or as µg·ml$^{-1}$.

In 22 subjects, we also compared iron content in the supernatant and cells obtained from the centrifugation of 10 ml of BALF.

Isolation of blood leucocytes

Peripheral venous blood was taken from eight healthy subjects (six with no exposure to industrial dust, and two iron-steelworkers) with the aim of separating lymphocytes, monocytes and neutrophils, for iron content comparison with alveolar macrophages. Blood (40 ml) was drawn into 10 ml Vacutainers® each containing 143 United States Pharmacopeia (USP) units of lithium heparin and 1 ml of Plasmagel (Bellon, Neuilly, France). The blood was gently mixed and allowed to sediment for 45 min at 37°C. The upper plasma layer, which is rich in leucocytes and platelets, was removed and pooled into a plastic tube. The concentration of leucocytes was adjusted with Hank's balanced salt solution without phenol red (HBSS Gibco) to a final concentration of 10$^6$ cells·ml$^{-1}$.

Mononuclear cells and neutrophils were separated by density gradient centrifugation of the leucocyte suspension on Lymphoprep® (Nyegaard, Oslo, Norway).
Aliquots of the leucocyte suspension (10⁷ cells·ml⁻¹) diluted 1/3 with HBSS without calcium and magnesium were layered into a 15 ml cushion of Lymphoprep (density 1.077) and centrifuged at 400 x g for 40 min at 22°C. The neutrophils were freed of red blood cells by hypotonic lysis using NH₄Cl. Mononuclear cells and neutrophils were separately washed twice with cold HBSS, without calcium and magnesium, containing 0.3 mg·ml⁻¹ human serum albumin, and resuspended in RPMI 1640 to 2x10⁶ cells·ml⁻¹.

To separate monocytes from lymphocytes, mononuclear cells (2x10⁶·ml⁻¹ in RPMI 1640) were cultured in 35 mm Petri dishes at 37°C in humidified air containing 5% CO₂. Non-adherent cells (lymphocytes) were removed after 2 h incubation and adherent cells were gathered (after 15 min incubation with 2 ml phosphate buffered saline (PBS) and ethylenediaminetetra-acetic acid (EDTA) at 4°C) with a rubber policeman. Adherent cells were at least 85% monocytes as judged by nonspecific esterase staining.

Histological characterization of iron (ferrocyanide reagent)

From the 98 cell smears stained by the ferrocyanide reagent, only those containing ≥85% macrophages were studied. Air-dried slides were stained for iron with the ferrocyanide reagent according to the following technique. This technique works well on the haemosiderin and most mineral iron found in macrophages [3-5]. The air-dried slides were fixed in absolute methanol for 5-10 min and then placed for 10 min in a fresh mixture (at 50-80°C) of equal parts of a 2% aqueous solution of potassium ferrocyanide and 4 N HCJ. Finally, slides were rinsed with distilled water and then counterstained 1-2 min with a 0.1% aqueous solution of eosin of sofrarin. By increasing the concentration of the hydrochloric acid to 4 N (instead of 0.1 N) and heating to 60-80°C, positive Prussian blue reactions may be obtained even with the more refractory ores [5]. The iron content of alveolar macrophages was quantified as follows: 200 macrophages were counted, and each cell was graded for iron on a scale of 0-4: 0=no blue colour; 1=faint blue staining in cytoplasm; 2=dense blue colour in minor portion of cytoplasm or medium colour intensity throughout the cell; 3=deep blue staining in most of the cytoplasm; 4=dark blue throughout the cytoplasm. A mean score for 100 cells was calculated; zero being the minimum and 400 the maximum score.

Table 1 shows BALF cytology and mean iron content of alveolar macrophages with respect to smoking habits in 43 healthy subjects without occupational exposure to industrial dust. We confirm that the alveolar macrophage population and iron content are significantly increased in smokers (p<0.05).

As shown in table 2, alveolar macrophages from workers occupationally exposed to iron dust such as welders and blast-furnace workers have an increased iron content in comparison with unexposed subjects (p<0.01). Surprisingly, coke-oven workers also have a high intramacrophagic iron content, although they are not directly exposed to iron dust (p<0.01).

The influence of pulmonary diseases in patients without occupational exposure to industrial dust is shown in table 3. Mean intramacrophagic iron is significantly increased in COPD and in lung cancer patients by comparison with healthy smokers or nonsmokers (p<0.01). As expected, the two patients with Goodpasture's syndrome had high iron content in their alveolar cells. Two retired steelworkers (a blast-furnace worker and a welder) have a high iron content in their alveolar cells.

Table 1. - BALF cytology and iron content of alveolar cells in 43 healthy subjects without occupational exposure to industrial dust

<table>
<thead>
<tr>
<th></th>
<th>Total n=43</th>
<th>Nonsmokers n=19</th>
<th>Smokers n=24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells×10⁶·ml⁻¹ BALF</td>
<td>15.1±13.8</td>
<td>6.7±3.8</td>
<td>21.7±15.2**</td>
</tr>
<tr>
<td>% macrophages</td>
<td>86.2±1.4</td>
<td>85.2±12.4</td>
<td>87.2±10.7</td>
</tr>
<tr>
<td>% lymphocytes</td>
<td>8.9±10.2</td>
<td>10.3±11.3</td>
<td>7.7±9.3</td>
</tr>
<tr>
<td>% neutrophils</td>
<td>2.7±2.4</td>
<td>2.8±2.6</td>
<td>2.7±2.2</td>
</tr>
<tr>
<td>% eosinophils</td>
<td>0.7±1.2</td>
<td>0.4±0.8</td>
<td>0.9±1.4</td>
</tr>
<tr>
<td>% bronchial cells</td>
<td>1.0±1.3</td>
<td>1.2±1.4</td>
<td>0.7±1.2</td>
</tr>
<tr>
<td>Fe μg·10⁶ cells</td>
<td>0.45±0.35</td>
<td>0.33±0.21</td>
<td>0.55±0.40*</td>
</tr>
<tr>
<td>Meantd</td>
<td>0.039-1.79</td>
<td>0.039-0.84</td>
<td>0.095-1.79</td>
</tr>
</tbody>
</table>

Nonsmokers versus smokers: *: p<0.05; **: p<0.01. BALF: bronchoalveolar lavage fluid.
worker and a welder) with siderosis had high amounts of iron in their pulmonary macrophages (2.6 and 5.24 µg·10^6 cells, respectively).

Figure 1 illustrates individual intramacrophagic iron values in the different subject groups.

As shown in figure 2, intracellular iron is significantly higher in alveolar macrophages than in blood leucocytes (monocytes, lymphocytes and neutrophils) from healthy subjects with and without occupational exposure to industrial dust (p<0.05).

There is a significant linear correlation between iron contents of the supernatant fluid and cells obtained by BALF centrifugation (fig. 3). About 80% of the total BALF iron is located in the cells rather than in the fluid phase. Multiple regression analysis applied to iron determination in alveolar macrophages by the ferrocyanide reagent's score and the PIXE method revealed a significant quadratic relationship (p<0.001) between the two techniques (fig. 4). The multiple correlation coefficient was 0.89 so that the

Table 2. - BALF cytology and iron content of alveolar cells in 106 healthy subjects according to occupational exposure to industrial dust

<table>
<thead>
<tr>
<th></th>
<th>Unexposed subjects</th>
<th>Blast furnace workers</th>
<th>Coke oven workers</th>
<th>Welders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron µg·10^6 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.45±0.35 (43)</td>
<td>1.77±1.53 (41)****</td>
<td>1.13±0.88 (19)**</td>
<td>1.68±0.58 (3)****</td>
</tr>
<tr>
<td>Smokers</td>
<td>0.55±0.40 (24)</td>
<td>2.06±1.74 (27)****</td>
<td>1.17±0.96 (14)**</td>
<td>1.98±0.27 (2)</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>0.33±0.21 (19)</td>
<td>1.45±1.05 (14)****</td>
<td>1.01±0.68 (5)</td>
<td>1.07 (1)</td>
</tr>
<tr>
<td>Cells x10^4·ml^1 BALF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Macro</td>
<td>86.3±11.4 (43)</td>
<td>16.2±11.2 (41)</td>
<td>23.0±16.8 (19)</td>
<td>14.2±14.6 (3)</td>
</tr>
</tbody>
</table>

Number of subjects in parentheses. Unexposed subjects versus blast-furnace workers, coke-oven workers and welders: **: p<0.01; ***: p<0.001. BALF: bronchoalveolar lavage fluid; Macro: macrophages.

Table 3. - BALF cytology and iron content of alveolar cells in 95 subjects with no occupational exposure to industrial dust according to pulmonary disease

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>COPD</th>
<th>Lung carcinoma</th>
<th>Goodpasture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron µg·10^6 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.45±0.35 (43)</td>
<td>2.06±0.86 (6)****</td>
<td>2.07±2.81 (44)****</td>
<td>6.22±1.52 (2)****</td>
</tr>
<tr>
<td>Smokers</td>
<td>0.55±0.40 (24)</td>
<td>2.06±0.86 (6)****</td>
<td>2.34±2.68 (27)**</td>
<td>5.15 (1)</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>0.33±0.21 (19)</td>
<td>1.64±3.03 (17)</td>
<td>1.64±3.03 (17)</td>
<td>7.3 (1)</td>
</tr>
<tr>
<td>Cells x10^4·ml^1 BALF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Macro</td>
<td>86.3±11.4 (43)</td>
<td>26.0±10 (6)</td>
<td>13.7±9.3 (44)</td>
<td>35±9.8 (2)*</td>
</tr>
</tbody>
</table>

Number of subjects in parentheses. Healthy versus COPD, lung carcinoma and Goodpasture's syndrome: *: p<0.05; **: p<0.01; ***: p<0.001. COPD: chronic obstructive pulmonary disease; BALF: bronchoalveolar lavage fluid.
percentage of variance explained was 80%. In comparison, for a linear regression line the percentage of variance explained was found to be 72%. Predicting iron results of the PIXE method by the ferrocyanide reagent’s score would be inappropriate, given the large dispersion of the observations around the regression curve.

![Graph](image)

**Fig. 3.** Correlation between iron content (µg) of the supernatant fluid and of the cells obtained by centrifugation of 10 ml BALF from 22 subjects. BALF: bronchoalveolar lavage fluid.

Our normal values of intramacrophagic iron in healthy unexposed subjects compare well with those reported previously by others [15, 16]. These values are, however, significantly lower than those obtained by Måås and co-workers [17-19] from rather heterogeneous populations containing, in addition to nonsmoking healthy subjects, smokers and patients with respiratory diseases. Clearly, there is still some uncertainty about normal iron content in alveolar cells and a need for better standardization of trace element determination in cells.

Our work confirms that intramacrophagic iron is significantly increased in smokers [15-17, 20]. Tobacco smoke, indeed, contains iron in its particulate phase. Ten percent of the mineral particles isolated from cigarette smoke are Fe-Ti particles (oxides), and most of them are inhalable [14]. An important point emerging from our study is that intramacrophagic iron is significantly increased in steelworkers and in patients with pulmonary diseases (COPD, lung cancer, Goodpasture’s syndrome). It was not surprising to find increased amounts of iron in retired workers who were exposed for years to iron dust (blast-furnace workers and welders) and in two workers with siderosis. By contrast, we have no definite explanation for the increase of iron in alveolar cells of coke-oven workers. Coke-oven smoke may contain mineral iron in addition to many irritants and carcinogens. Chronic exposure to coke-oven fumes and gases may lead to chronic bronchitis and nonspecific bronchial hyperresponsiveness [21, 22]. It can be speculated that in healthy coke-oven workers...

![Graph](image)

**Fig. 4.** Correlation between iron determination in alveolar macrophages (macro) by the ferrocyanide reagent’s score and the PIXE method. Healthy subjects (○) and patients (●) without occupational exposure to industrial dust, and healthy subjects (△) and patients (▲) with occupational exposure to industrial dust. PIXE: particle-induced X-ray emission.

**Discussion**

Iron determination in bronchoalveolar lavage cells and fluid via the PIXE method allows the measurement of organic (iron binding proteins, haemoglobin, haemosiderin) as well as mineral iron which may be inhaled from the urban [12, 13] or occupational environment. Our results indicate that the mean iron content of washed alveolar cells (87% macrophages) of nonsmoking healthy subjects is 0.33±0.21 µg·10⁶ cells. This value is significantly higher than that obtained from autologous blood leucocytes. The high iron concentration in alveolar macrophages in comparison with blood leucocytes may be explained by chronic inhalation of iron particles present in natural [12, 13] or occupational air and in cigarette smoke [14]. Alternatively, it may reflect some intrinsic structural differences between alveolar macrophages and monocytes [1].
workers some degree of bronchial inflammation may occur and cause extravasation of iron-binding proteins, alveolar macrophage activation and a decrease of mucociliary clearance with particle retention.

The increase of iron in alveolar macrophages of COPD and lung cancer patients can not be accounted for by cigarette smoking alone. Here again, it may be speculated that chronic airway inflammation, mucociliary clearance reduction and microhaemorrhage, could partly explain the high intramacrophagic iron load observed in these patients. It is known that chronic inflammation in other organs is associated with local and systemic alterations in iron metabolism. For example, in rheumatoid arthritis, reticuloendothelial stores are increased, whereas serum iron, transferrin and transferrin saturation are all reduced [2]. Serum ferritin concentration is often at the upper limits of normal values and may be slightly higher [23]. Increased amounts of iron have been found in the synovial macrophages in inflammed joints [24]. A similar situation could occur in patients with airway diseases and coke-oven workers.

The finding of high intramacrophagic iron in the two patients with Goodpasture's syndrome with no known occupational exposure was expected and can be logically explained by patent or occult pulmonary haemorrhage. Occult pulmonary haemorrhages may be detected with a computerized tomographic (CT) scan or by a high carbon monoxide transfer [25], but they are more reliably diagnosed with the ferrocyanide reagent's score [6, 7].

We conclude, that intramacrophagic iron may be increased in steelworkers and subjects with pulmonary haemorrhage, but also in asymptomatic smokers, in COPD and in lung cancer patients without occupational exposure to iron dust.

References