An attempt to evaluate lung aggression in monkey silicosis: Hydrolases, peroxidase and antiproteases activities in serial bronchoalveolar lavages


ABSTRACT: Exposure to silica can induce fibrosis and/or emphysema. Various factors such as proteases, other hydrolases and oxidants may be involved in the destruction of lung parenchyma. On the other hand, antiproteases play an important role in the protection of lung parenchyma against the action of proteases. We have developed an animal model of silicosis in monkey Macacus cynomolgus and followed these factors by bronchoalveolar lavage (BAL). We have studied glycosidases activities, elastase-like activity, immunoreactive α1-protease inhibitor (α1PI), neutrophil elastase inhibitory capacity (NEIC) and myeloperoxidase. Bronchoalveolar cells in serial BAL were also studied. Six monkeys were exposed to quartz aerosols (100 mg ·m·⁻³) for 18 wks. They were followed until they developed X-ray changes, which occurred between 21–64 wks after the end of the dust exposure. Cellular "silicotic nodules" were observed in lung biopsies. A control animal underwent serial BAL. Changes were seen in the differential cell count. The release of superoxide anion by bronchoalveolar cells obtained during the experiment was increased. Separation on a gradient of Percoll showed the presence of young macrophages, which exhibited enhanced release of superoxide anion as compared to the totality of bronchoalveolar cells. The biochemical analysis of BAL fluids obtained during and after the period of dust exposure showed an increase in glycosidases, α1PI and NEIC. Some free elastase-like activity was simultaneously detected in BAL fluids from exposed animals but not from the control. This elastase-like activity was very low compared to NEIC. The increase in enzymatic and antiprotease activities occurred at different points in time for each animal, suggesting large differences in individual responses to dust, but occurred before the chest X-ray abnormalities.


Silicosis is a fibrotic lung disease resulting from the inhalation of quartz or silica. Silica induces pulmonary inflammation and fibrosis and/or emphysema [1–3] but very little is known about the early pathophysiological events leading to the development of silicosis.

Since silica can induce fibrosis and emphysema [4–5], pathological syndromes where various factors such as imbalance between proteases and antiproteases [6, 7] and/or oxidants [8] may play a role. We have developed an animal model of silicosis allowing the follow-up of these various aggressive factors by bronchoalveolar lavages.

A monkey, Macacus cynomolgus, was chosen to create this experimental model for two reasons: i) these animals can be submitted to repeated lavages and it is possible to follow the course of the events over a long period of time; and ii) even if this experiment could not be performed on a large number of animals, the monkey model is interesting because of its phylogenetic relationship to humans.

The follow-up of enzymes which are able to destroy pulmonary tissue may be interesting. These enzymes may originate from neutrophils and/or macrophages, or from interstitial cells [3, 5]. Leucocyte elastase can solubilize lung elastin [9]. Lung collagen can be
destroyed by collagenase and also by leucocyte elastase [10]. But destruction of lung connective tissue involves the combined effects of many enzymes, and the action of proteinases might be completed by that of lysosomal enzymes such as glycosidases which are able to catabolize glycoproteins [11]. Therefore, we decided to study an elastase-like activity and three glycosidases, N-acetyl-β-D-glucosaminidase, N-acetyl-β-D-galactosaminidase and β-D-galactosidase.

Under physiological conditions, antiproteases may play an important role in the protection of lung parenchyma against the action of proteases. We have focused our study on α1-protease inhibitor (α1PI) which has been described as the major antiprotease at the alveolar level [7].

Recent research has also focused on oxidant mediated lung injury. Oxidants are able to destroy many elements of the alveolar wall [8]. Moreover, oxidants can inactivate antiproteases, thereby enhancing the proteolytic activity of proteinases [12]. Oxidant activity may be due either to enzymes such as myeloperoxidase or to oxygen free radicals such as superoxide anion secreted by phagocytic cells [2, 8]. We decided to study superoxide anion released by bronchoalveolar cells, and to look for the presence of myeloperoxidase in BAL fluids.

Since silica induces an accumulation of inflammatory cells within the alveolar structure [2], we have compared the evolution of the hydrolases, peroxidase and antiproteases with the modification of the bronchoalveolar cells in serial fluids.

The different measurements were carried out on bronchoalveolar lavages (BAL) obtained before, during and after dust exposure of six monkeys and were completed by chest X-rays and histopathological examination of lung tissue.

The ultimate goal of the present work was to find out if any of these cellular, enzymatic or antiproteases activities could have a predictable value and be useful to follow the early pathological process due to dust inhalation.

Material and methods

Design of the experiment

Seven female Macacus cynomolgus monkeys numbered I to VII were used in this study. They were 5 yrs old at the beginning of the experiment. They weighed between 2,700 and 4,360 g. These animals were provided from Station Centrale de Physiologie Animale de l’INRA. They were first accustomed to laboratory conditions for two months. Six of them were then exposed to inhalation of respirable dust of natural quartz (Madagascar) at a concentration of 100 mg·m–3, 4 h a day, 5 days a wk for 18 wks. Inhalation facilities and dust generating techniques have been described in detail [13]. The seventh animal was used as a control and kept in similar conditions but without dust exposure.

Animals underwent chest X-ray under anaesthesia every month, during and after exposure period (post-exposure period). They were followed until the detection of chest X-ray changes, characterized by reticulo-micronodular shadows in the perihilar region. Each of them had lavages before, during and after exposure period. Three lavages were performed prior to exposure. During the exposure period, lavage was carried out on weeks 6, 10 and 18, it was done at almost monthly intervals in the post-exposure period. Control animals was also submitted to repeated lavages and to chest X-rays.

Bronchoalveolar lavage

After general anaesthesia with 100–150 mg of ketamine hydrochloride (Rhône-Merieux-Lyon-France) and local anaesthesia with a puff of lidocaine (Roger Bellon, Neuilly sur Seine, France) animals were intubated with an endotracheal cannula (Portex 3.5 or 4) and ventilated with an RPR ventilator. Lavages were performed via a soft-type cannula passed through the endotracheal tube and positioned in one of the lower lobes. The lavages were performed under fluorescence X-rays. Five ml aliquots of 0.9% saline were infused and then gently aspirated. This was done 10 times. In order to minimize bronchial contamination, the first 5 ml were separated. The other tubes were pooled and centrifuged at 800 g for 10 min. The supernatant fluid was frozen until used.

Cell analysis

The cell pellet obtained after centrifugation of the bronchoalveolar lavage was washed twice with Hank’s medium. The cells were counted in a haemocytometer chamber and expressed as the number of cells for the totality of the recovered fluid. Differential counts were performed on cytospin smears (Shandon Sander Corporation, Runcorn, England) stained by the May-Grünwald method.

Bronchoalveolar cells were fractionated using a discontinuous gradient of Percoll according to Elias et al. [14]. 3×10⁶ cells were layered on an 8 ml gradient containing 2 ml each of Percoll specific gravity (1.035, 1.044, 1.057, 1.072). The gradient was then centrifuged at 800 g for 30 min at 4°C. The cells that were localized at each gradient interface were collected, washed twice in Hank’s medium and resuspended in the same medium. The different alveolar macrophage subpopulations were counted and submitted to cytospin centrifugation in order to determine the differential cell count.

The amount of superoxide released by bronchoalveolar cells was determined using a Lucigenin dependent chemiluminescence method according to Williams and Cole [15]. We measured spontaneous and phorbol myristate acetate (PMA) induced chemiluminescence. Each experiment was performed in the presence and absence of superoxide-dismutase (SOD).
Lucigenin (Sigma Chemical Co; St Louis, Missouri, USA) was dissolved in Hank's medium (2 mg·ml⁻¹). Phorbol myristate acetate (PMA, Sigma) was dissolved in dimethylsulphoxide (1 mg·ml⁻¹) and diluted 1/5000 in Hank's medium prior to use. Superoxide dismutase (Sigma) was dissolved in water (2 mg·ml⁻¹).

Chemiluminescence was measured at 37°C using a six channel Packard Luminoimeter. Cells were resuspended at a concentration of 1×10⁶ cells·ml⁻¹ in Hank's medium. 250 µl of cell suspension was added to each vial and then 0.9 ml of lucigenin. The vials were then placed in the carousel of the luminometer. In some vials 100 µl of PMA was added. Spontaneous and PMA-induced chemiluminescence were also measured in the presence of 50 µl of SOD solution in order to discriminate chemiluminescence due to superoxide anion generation. It represented chemiluminescence inhibitable by SOD.

For each vial chemiluminescence was counted for 25 s at 2 min interval. It was followed kinetically during 10 min. Results were expressed in relative luminescent units (RLU) per number of viable cells in 1 ml of recovered BAL fluids.

Biochemical analyses in bronchoalveolar lavage fluids

Glycosidase activity, myeloperoxidase activity, elastase-like activity, neutrophil elastase inhibitory capacity and immunoreactive α-protease inhibitor were measured in the BAL supernatant. Methods are described below; all measurements were repeated twice and produced similar results. They were expressed as per ml of BAL fluid.

Glycosidase activity was measured, using methylumbelliferyl glycosides as substrates as described previously [16]. Enzymatic activities were expressed as nanomoles of substrate liberated per min (arbitrary unit).

Myeloperoxidase activity was measured by determining the change in absorbance of O-dianisidine (Sigma Chemical) as described previously [16, 17]. Horseradish peroxidase (Sigma Chemical) was used for calibration purposes. Peroxidase activity in BAL fluid was expressed as mU·min⁻¹.

 Amidolytic activity was determined on the synthetic substrate of elastase succinyl-(trialanyl)-para-nitroanilide (SLAPN) (Biosys, Compiegne, France) [18]. This activity was referred to as free elastase-like activity. It was quantified after 2 h incubation at 37°C with BAL fluids. The activities of dilutions from a solution of neutrophil elastase were determined and compared to those of BAL fluids. Neutrophil elastase was purified from piglet sputum [19] and found to be more than 90% active by active site titration using published kinetic constants [20]. Elastase-like activity in BAL fluids was expressed as neutrophil elastase equivalent (10⁻¹⁰ mol·ml⁻¹·min⁻¹).

The measurement of neutrophil elastase inhibitory capacity (NEIC) was based upon the hydrolysis of L-pyroglutamyl-L-prolyl-L-valine-para-nitroanilide (LS 2484, Kabi Vitrum Diagnostic, Stockholm, Sweden) [21]. The stock solution (100 mM in dimethyl sulphoxide (DMSO) was diluted prior to use at a concentration of 15 mM in microtitration plates (Nunc, Roskilde, Denmark) coated with serum albumin. The stock solution of neutrophil elastase (3.5×10⁻⁵ M in sodium acetate buffer 0.05 M, pH 5.5, NaCl 0.45 M) was diluted and ten microlitres (3.5×10⁻⁹ moles) were incubated with 40 µl of phosphate buffer 0.25 M, pH 7.5, NaCl 1 M, and with increasing amounts of BAL fluid (0 to 140 µl). The final volume was made up to 190 µl with normal saline. After 15 min at room temperature, 10 µl (1.5×10⁻⁷ mol) of substrate were added. The mixture was then incubated for 30 min at room temperature. The reaction was stopped by addition of 100 µl of a 50 μl·100 ml solution of acetic acid. Residual enzymatic activity was measured by the optical density readings at 405 mm with a Titertek-Uniscan II (Flow Laboratories, Puteaux, France). Neutrophil elastase inhibitory capacity (NEIC) was calculated from the point of functional equivalence (determined by linear regression) and expressed as 10⁻¹⁰ moles of elastase inhibited per ml of BAL fluid.

Immunoreactive α-protease inhibitor (α,PI) was determined by laser immunonephelometry as described previously [22]. A Hyland-laser nephelometer PDQ (Hyland Laboratories, Garden Grove, USA) was used in accordance with the procedures recommended by the manufacturer (Hyland α,PI-antibody and Hyland-test standard A). The results were expressed as 10⁻¹⁰ moles of human α,PI equivalent per ml of BAL fluid. Previous studies have shown that monkey α,PI cross-reacted with human antiserum against human α,PI [16].

Silica contents of BAL fluids

BAL fluids were passed through a membrane filter (Millipore 0.2 µm). Silica content was determined by infrared spectrophotometry [23]. The results were expressed as µg of silica·ml⁻¹ of BAL fluid.

Lung biopsies

All the silica exposed animals had a lung biopsy of the right middle lobe in the post-exposure period but one died during the period of observation and lung samples were taken at autopsy. The other animals had a lung biopsy at wk 30 for animals VI, at wk 22 for animals II and III, at wk 40 for animals IV and V. Lung biopsy was obtained by right posterolateral thoracotomy performed under general anaesthesia (100 mg ketamine hydrochloride). Samples were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. (4–5 µm). Serial sections obtained from the whole tissue fragments, were stained with haematoxylin and eosin, sirius red and Gordon Sweet [24]. For each animal the intensity of the histological lesions was scored on a 0–5 scale, according to the criteria presented in table I [25].
Table 1. – Grading of histological lesions [25]

<table>
<thead>
<tr>
<th>Intensity grade</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal histology</td>
</tr>
<tr>
<td>1</td>
<td>Granulomas with reticulin fibres located in periphery of interspersed throughout the lesion. These granulomas were predominantly adjacent to vessels and respiratory bronchioles. They were surrounded with inflammatory cells, especially macrophages.</td>
</tr>
<tr>
<td>2</td>
<td>Early collagenous organization of the granulomous with reticulin fibres and peripheral thin collagen bundles.</td>
</tr>
<tr>
<td>3</td>
<td>Nodular fibrotic reaction. Granulomas with meshwork of collagen fibres in concentric arrangement.</td>
</tr>
<tr>
<td>4</td>
<td>Confluence of the type III collagenous nodules.</td>
</tr>
<tr>
<td>5</td>
<td>Massive fibrotic reaction, consisting of dense hyaline collagenous fibrosis.</td>
</tr>
</tbody>
</table>

Statistical studies

Statistical studies were performed on whole BAL obtained during and after dust exposure, the results were compared to those of BAL obtained before dust exposure.

For technical reasons it was not possible to perform BAL at exactly the same time for all the animals. In order to perform statistical studies, we have studied the period when BAL were available for all the animals.

During dust exposure BAL were studied at 3 periods corresponding to 6 wks exposure (D1), 10 wks exposure (D2), 18 wks exposure (D3). The post exposure period was divided in 5 sequences of time called respectively, PE (4–9 wks), PE, (13–17 wks), PE, (20–25 wks), PE, (29–39 wks). After that period not enough BAL were available.

Group data are expressed as a mean±sem. Analysis for variable parameters was studied by the Kruskall-Wallis test.

Results

The control animal (animal VII) did not show any chest X-ray abnormalities. A lung biopsy taken at wk 55 was normal.

As already indicated, one animal (number I) died at wk 29 after the end of the exposure. It exhibited chest X-ray changes at wk 21. Histological examination of the lung at autopsy revealed a massive fibrotic reaction (fig. 1), infiltration of inflammatory cells and the presence of microabscesses. Emphysematous lesions were also observed.

The five other animals (nos II–VI) exhibited chest X-ray changes at wk 32, 39, 52, 59 and 64, respectively. Histological abnormalities consistent with a pathological process due to dust inhalation were progressively observed in all animals. Animal VI presented only slight histological modification (grade 1) at wk 30. Grade 2 was observed at wk 22 for animals II (fig. 2) and III. Grades 3 and 4 were observed at wk 40, respectively, for animals V and IV. So called "silicotic nodules" were present in more advanced lesions, all were cellular.

Cell analysis

The results obtained for control animal (monkey VII) are reported in table 2. We did not notice important variation in the number of total cells, the differential cell count was not modified. Similar results have already been described for control monkeys submitted to repeated lavages [16].

For the exposed animals (monkeys I to VI) the number of total cells was not considerably increased in BAL fluids obtained during and after dust exposure (data not shown).

Fig. 1. – Lung histology of animal I (autopsy sample at wk 29). A: Massive fibrotic reaction with dense, poorly cellular collagen areas grade 5 (haematoxylin and eosin ×60). B: Dense collagenous tissue (Gorden and Sweet ×40).
Fig. 2. – Lung histology of animal II at wk 22. A: Cellular granulomatous reaction grade 2 (haematoxylin and eosin x60). B: Early collagenous organization of the granulomas (Gorden and Sweet x150).

Table 2. – General characteristics of serial BAL fluids obtained from control animal (monkey)

<table>
<thead>
<tr>
<th>Cell analysis</th>
<th></th>
<th>n=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell counts for the totality of the recovered fluid ($\times 10^6$)</td>
<td>2.42±0.4</td>
<td></td>
</tr>
<tr>
<td>Differential cell count % Macrophages</td>
<td>91.4±3.4</td>
<td>(88-97)</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>1.12±1.26</td>
<td>(0-4)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.86±3.02</td>
<td>(2-10)</td>
</tr>
<tr>
<td>Mastocytes</td>
<td>1.12±1.26</td>
<td>(0-4)</td>
</tr>
<tr>
<td>Superoxide anion release (RLU $\times 10^6$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>0.42±0.34</td>
<td>(0.10-1.9)</td>
</tr>
<tr>
<td>PMA-induced</td>
<td>0.88±0.45</td>
<td>(0.4-1.9)</td>
</tr>
</tbody>
</table>

Biochemical analysis of BAL fluids

| N-acetyl-$\beta$-D-glucosaminidase | 1.98±0.52 | n=11 |
| Elastase like activity            | 0         |      |
| $10^{-11}$ mol·ml$^{-1}$          |           |      |
| Immunoreactive $\alpha$PI $10^{-11}$ mol·ml$^{-1}$ | <1 | n=3 |
| NEIC $10^{-11}$ mol·ml$^{-1}$     | 2.1±0.67  | n=11 |
| (0.4-1.9)                        |           |      |

The values are expressed as mean±s.p. Range in parentheses. NEIC: neutrophil elastase inhibitory capacity; PMA: phorbol myristate acetate; BAL: bronchoalveolar lavage; RLU: relative luminescent units.

Modifications were obtained for the differential cell count (fig. 3). The differential count was not done for monkeys I and V in control BAL fluids, but previous results [16] have shown that the control cellular population is composed of 88% macrophages, the others cell being principally lymphocytes.

The modifications of the differential cell count occurred mainly during the dust exposure period. At that time the percentage of macrophages was decreased, the values obtained averaged 60% of the total cell count, a concomitant increase in the percentage of lymphocytes was observed (fig. 3). An important increase in the percentage of leucocytes was observed during the dust exposure period, the values ranged between 15-30% of the total cell count. During the post exposure period the values remained higher than those obtained in lavages before dust exposure. An increase in the percentage of mastocytes occurred during the post-exposure period for animals I, II and IV, in these lavages the percentage of mastocytes reached 12%, while in control BAL fluids the percentage of mastocytes had an average value of 4%.

When control bronchoalveolar cells were fractionated on a discontinuous gradient of Percoll, three fractions were obtained (at d=1.035, 1.044, 1.057) according to their size called, respectively, cells 1, 2 and 3. Bronchoalveolar cells obtained during and after dust exposure gave a fourth fraction (cells 4) at d=1.072; those cells were morphologically similar to monocytes (data not shown).

Lucigenin dependent chemiluminescence was mainly due to superoxide anion release, since 94% of chemiluminescence was inhibited by SOD.

For the control animal the results obtained are presented in table 2. Despite some variations, from one lavage to another, the values of spontaneous and PMA induced superoxide release were not increased during the course of the different lavages. Furthermore, variations were not dependent on the time the lavage was performed.

For exposed monkeys the results obtained for spontaneous and PMA-induced superoxide release are represented in fig. 4. Individual variations in the time
course and magnitude of response were observed for the six animals.

Spontaneous superoxide anion release was not modified during the course of the experiment. When cells were incubated with PMA superoxide anion release was increased during dust exposure period for monkeys I, II and V, the increase averaged a factor 2. During the post-exposure period, the increase occurred at different points of time, before chest X-ray changes for monkeys I, II and simultaneously for animals IV and V. Furthermore, the magnitude of increase was also different from one animal to another. For monkeys I, II and IV RLU were increased by a factor 10, while RLU was increased 2 and 3 folds for animal V. The value of RLU were not increased for animals III and VI.

In some experiments it was possible to perform chemiluminescence studies on cells fractionated by the gradient of Percoll. A typical result is presented in fig 5. The values of RLU of cells 4 were higher than those obtained for cells 1, 2 and 3 and for native cells.
Fig. 4. - Lucigenin dependence of cells obtained in serial bronchoalveolar lavages. C: control period; D: dust exposure period; PE: post-exposure; R+: X-ray changes. RLU: relative relative luminescent units. The results were expressed for the totality of cells in 1 ml of recovered BAL fluid. - - - : spontaneous; — : PMA-induced.

Table 3. - Silica content in serial BAL fluids

<table>
<thead>
<tr>
<th></th>
<th>Animal I</th>
<th>Animal II</th>
<th>Animal III</th>
<th>Animal IV</th>
<th>Animal V</th>
<th>Animal VI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dust exposure wks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.98</td>
<td>0.21</td>
<td>0.29</td>
<td>0.08</td>
<td>0.88</td>
<td>0.34</td>
</tr>
<tr>
<td>10</td>
<td>2.89</td>
<td>0.16</td>
<td>0.43</td>
<td>0.30</td>
<td>0.34</td>
<td>0.15</td>
</tr>
<tr>
<td>18</td>
<td>0.48</td>
<td>0.13</td>
<td>0.35</td>
<td>0.20</td>
<td>0.54</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Post-exposure period wks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–10</td>
<td>0.64</td>
<td>0.12</td>
<td>0.17</td>
<td>0.07</td>
<td>0.27</td>
<td>0.17</td>
</tr>
<tr>
<td>11–20</td>
<td>0.13</td>
<td>0.06</td>
<td>0.06</td>
<td>0.04</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td>21–30</td>
<td>0.07</td>
<td>0.04</td>
<td>0.08</td>
<td>0.07</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Silica content is expressed as mg·ml⁻¹.
Silica content of BAL fluids

The results obtained during and after dust exposure are reported in table 3. Important variations were obtained from one animal to another, very high values were obtained from animal I. For all animals the higher values were found during dust exposure period. In the post-exposure period, after 30 wks the silica content was very low (<0.04 mg·ml⁻¹).

Biochemical analysis of BAL fluids

The results of the measurement of hydrolases and antiproteases activities in sequential bronchoalveolar lavages are reported in table 2 for the control animal and in fig 6 and 7 for exposed monkeys.

Fig. 6. - N-acetyl-β-glucosaminidase (——) and elastase-like activity (•••••••) in serial BAL fluids. C: control period; D: dust exposure period; PE: post-exposure; R+: X-ray change.
For the control animal, the values did not change in the different lavages and over the time of the experiment. The results are very close to those previously obtained in controls animals submitted to repeated lavages [16].

For exposed monkeys results of the measurements of hydrocolases and antiproteases activities are reported in figures 6 and 7. Despite large individual variations in the time course and magnitude of biochemical changes, some systematic patterns were observed.

N-acetyl-β-D-glucosaminidase was increased during dust exposure for animals I, II and VI (fig. 6). During the whole post-exposure period an increase was noticed for animals I, II and III. For animal I, the values obtained after 17 wks of post-exposure period reached 75 units, they were increased by a factor 10 when compared to the values obtained before dust exposure. For animals II and III, a continuous and slight increase was shown over the whole post-exposure period, the values ranged between 5–9 units. For monkeys IV, V,
The variations of peroxidase were also determined for all the animals (data not shown). In control BAL fluids, the values of peroxidase ranged from 0–0.2 units. A fivefold increase was obtained for animal I, during dust exposure; for the other animals the values were very closed to those obtained in control BAL fluids. The same results were obtained for all the animals, in the post-exposure period.

**Statistical studies**

The results are reported in table 4. For N-acetyl-\(\beta\)-D-glucosaminidase and NEIC the values obtained during and after dust exposure were significantly higher than those obtained prior to dust exposure (\(p<0.05\) or \(p<0.01\)). For \(\alpha_1\)PI the values obtained during the experiment were higher than the control values, however, the differences were not significant.

**Discussion**

In this study, X-ray changes were produced in monkeys exposed to inhalation of quartz. These changes occurred between 21–64 wks after the end of the exposure, suggesting a significant individual reaction to dust inhalation. Histological studies confirmed a pathological process due to dust inhalation. The lesions obtained were similar to those usually described in silicosis, such as silicotic nodules, granulomas with inflammatory cells. For animal I, a massive fibrotic reaction was described, but it was not found proteinosis features of acute silicosis [3]. The intensity grade of histological lesions were also variable from one animal to another. These results are also in favour of an individual reactivity to silica.

Different reaction to dusts have already been described in man [1]. Beauv et al. [26] have also demonstrated an individual heterogeneity of lung tissue in a sheep model of asbestosis. For these authors it was related to the individual capacity of dust alveolar clearance. In our study the content of silica in BAL fluids was different from one animal to another. Animal I, which presented early abnormalities of chest X-rays, had a high silica concentrations.

**Table 4. – N-acetyl \(\beta\)-D-glucosaminidase, \(\alpha_1\)PI and NEIC in serial BAL fluids**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D₁</th>
<th>D₂</th>
<th>D₃</th>
<th>PE₁</th>
<th>PE₂</th>
<th>PE₃</th>
<th>PE₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-(\beta)-D-glucosaminidase units</td>
<td>2.12±1.15</td>
<td>11.76±12.24</td>
<td>15.73±12.87</td>
<td>5.95±2.16</td>
<td>3.79±1.75</td>
<td>8.59±9.11</td>
<td>14.24±24.33</td>
<td>5.04±2.36</td>
</tr>
<tr>
<td>(\alpha_1)PI 10⁻¹¹ mol·ml⁻¹</td>
<td>2.03±1.28</td>
<td>3.47±3.29</td>
<td>4.03±3.47</td>
<td>2.81±1.23</td>
<td>2.63±1.54</td>
<td>3.47±3.13</td>
<td>3.82±3.87</td>
<td>3.96±2.58</td>
</tr>
<tr>
<td>NEIC 10⁻¹¹ mol·ml⁻¹</td>
<td>2.45±1.69</td>
<td>5.08±3.68</td>
<td>6.79±6.79</td>
<td>5.85±2.11</td>
<td>5.35±4.93</td>
<td>11.74±13.02</td>
<td>12.6±17.32</td>
<td>6.51±2.9</td>
</tr>
</tbody>
</table>

*: \(p<0.05\); **: \(p<0.01\); NS: not significant; C: control period; D: dust exposure; PE: post-exposure; NEIC: neutrophil elastase inhibitory capacity; BAL: bronchoalveolar lavage; \(\alpha_1\)PI: alpha-1-protease inhibitor. Values are expressed as mean±SEM. During and after dust exposure \(n=6\), except for PE, \(n=5\). For the control period \(n=18\).
content in BAL fluids obtained during dust exposure. However, there is no evident relationship between silica content of BAL fluid and pulmonary functions such as alveolar clearance.

For all the monkeys submitted to dust, differences were obtained in the differential cell count and in superoxide anion release. In BAL fluids the values of glycosidases and elastase-like activity, α,PI and NEIC were increased in lavages obtained during and after dust exposure. We previously reported that cellular population hydrolases, peroxidase and antiprotease activities in BAL fluids were not affected by repeated lavages in unexposed monkeys [16].

In this study the number of total cells recovered during and after dust exposure was not increased; these results are different from those usually obtained in experimental silicosis [27–29]. This discrepancy may be explained by the fact that, in our study, exposure to silica was performed by inhalation while intratracheal instillations of silica were used in previous studies [27–29].

For all the animals the differential cell count was modified especially during dust exposure. The percentage of leucocytes and lymphocytes was increased, while the percentage of macrophages was decreased.

The results concerning leucocytes are very similar to those obtained by other authors in experimental silicosis [27–29]. Recruitment of leucocytes in the lung, has been described as an early response to silica and plays an important role in the inflammatory process [28].

Divergent results have been described concerning the proportion of lymphocytes and macrophages obtained during experimental silicosis. DAUBER et al. [29] found a decrease in the percentage of macrophages and no change for lymphocytes while BEGIN et al. [25] found an increase of macrophages. In our experiment, a decrease in the percentage of macrophages and a concomitant increase in the percentages of lymphocytes were obtained, at the beginning of the dust exposure period.

We have measured superoxide anion release by bronchoalveolar cells of serial bronchoalveolar lavages. The spontaneous release of superoxide anion was not increased during dust exposure. CANTIN et al. [30] using intratracheal instillation of asbestos or silica, also did not obtain an important release of superoxide anion [30]. However, bronchoalveolar cells obtained during our experiment released much higher level superoxide in the presence of PMA, than did control cells. These results indicate that exposure of the lower tract to silica markedly enhances the capacity of alveolar phagocytes to release superoxide in response to a membrane stimulus. This result may be important since reactive oxygen intermediate plays an important role in the destruction of lung parenchyma [8]. The increase of PMA-induced superoxide anion release did not occur at the same time for all the monkeys. An early increase was obtained for the animals which presented early X-ray changes suggesting a heterogeneous response of the cells to dust.

The heterogeneity of the macrophage population obtained by bronchoalveolar lavage has been described by several authors [5, 14]. Silica can induce an efflux of new cells in the lung. Using a discontinuous gradient of Percoll, we have demonstrated the appearance of a new population of macrophages in BAL fluids obtained during and after dust exposure. These cells had the appearance of young macrophages. Furthermore, they exhibited an enhanced release of superoxide anion as compared to the more mature cells, or to the unseparated population. These results may support the hypothesis that the augmented release of superoxide anion obtained in our experiment was related to the maturity of newly arrived cells in the lung following silica inhalation. These findings may be of importance in the pathological process due to inhalation, since oxygen intermediate plays a role in the destruction of lung parenchyma [8]. Furthermore, newly arrived macrophages also secrete more enzymes than more mature cells [5].

The results obtained in serial BAL fluids showed that peroxidase was not increased in BAL fluids obtained during and after dust exposure. An increase of this enzyme has been described after intratracheal instillation of chemotactic peptides [31], so peroxidase might eventually be only a marker of acute injury.

On the contrary, glycosidases and elastase-like activity, α,PI and NEIC were increased during and after dust exposure, but with large inter-animal variations. Such variations were already reported by CALLIS et al. [28] who have shown that response of mice to free silica was strain-dependent. For most animals an increase in hydrolases and antiproteases was observed during dust exposure followed by a drop after termination of exposure. Similar results have already described in experimental silicosis [27–29]. This increase may be transient indicating an early response to heavy silica exposure.

During the period of evolution, modifications of the biochemical parameters occurred either at early stages indicating a fast response to dust for animals I, II and III or at later stages indicating a later response to dust for animals IV, V and VI. These results are in good agreement with the follow-up of chest X-rays. Modifications of biochemical parameters generally occurred before the occurrence of chest X-ray change.

Among the aggressive factors studied, an important increase in glycosidases was found; furthermore, a slight but constant increase of an elastase-like activity was also detected. This elastase-like activity was found despite an increase of α,PI and NEIC which play an important role in the protection of lung parenchyma. High level of glycosidases have already been characterized in BAL from animals with experimental silicosis [32, 33]. Glycosidases possess biological activities against many of the structural components of pulmonary tissue [11], therefore, they may be of importance in many lung diseases such as silicosis.
Glycosidases may originate from macrophages activated by silica [3, 5]. Since we did not find an increase in the number of total free cells, it is possible that glycosidases were related to the state of activation of the free cells obtained by BAL. These cells are activated as shown by the increased release of superoxide anion. Furthermore, glycosidase activities may also be related to young populations of macrophages obtained during dust exposure [5]. This population of cells release more superoxide anion than more mature cells, the same phenomenon may occur for glycosidase. Finally, glycosidases may also be secreted by other alveolar cells such as pneumocytes type II [34] and leucocytes [35].

The presence of $\alpha_1$PI in BAL fluids is dependent on its plasma concentration and on the degree of pulmonary inflammation leading to an increased transudation [36]; thus, it may be an indirect marker for an inflammatory process. This protein which is considered as the major anti-elastase of the BAL fluid [7] was slightly increased before the occurrence of abnormalities of chest X-rays for most of the animals. However, when statistical studies were performed no significant difference were found. But the role of $\alpha_1$PI depends on its functional activity, therefore, we have determined its functional activity by measuring neutrophil elastase inhibitory capacity. NEIC was increased in BAL fluids obtained during and after dust exposure. In control BAL fluids and during dust exposure the values of $\alpha_1$PI and NEIC were very similar (mol/mol), while in BAL fluids obtained in the post-exposure period the values obtained for NEIC were higher than the values obtained for $\alpha_1$PI. The complex between $\alpha_1$PI and neutrophil elastase has been described to be 1 mol/l mol in human. Since monkey $\alpha_1$PI is chemically and antigenically very similar to that of human [16, 37] it might be assumed that the complex is also mol/mol. Therefore, the discrepancies between the values obtained for $\alpha_1$PI and NEIC suggest that, in our monkey model, dust exposure induces the synthesis of other inhibitors. These inhibitors have not, yet been characterized in monkey BAL fluids; however in human BAL fluids several other inhibitors such as the "bronchial inhibitor" have been described [38, 39]. These inhibitors may play an important role in the prevention of lung destruction by proteases.

Despite important NEIC, an amylolytic or elastase-like activity not detected in control BAL fluids was characterized in all BAL fluids obtained during the experiment. Such an activity has already been described in BAL fluids from smokers [40]. It should be emphasized that the values obtained for this activity were very low when compared to NEIC. Furthermore, this activity was determined on a synthetic substrate, and we do not know if it is active on elastin. Several hypotheses may be proposed for the presence of elastase-like activity in BAL fluids. Firstly, this enzymatic activity may originate from the dissociation of an enzyme/inhibitor complex, for instance the neutrophil elastase/$\alpha_1$PI complex as described by Jochum et al. [41]. An elastolytic activity may be also complexed with $\alpha_2$ macroglobulin but in this case elastase-like activity is only active on small synthetic substrates [42].

In the present study the comparison of the findings of chest X-rays, histological studies and the results of serial bronchoalveolar lavages allows the distinction of two groups of animals. A first group has a fast response to dust as shown by early changes in chest X-rays. For these animals modifications of hydrolases, antiproteases and superoxide anion release occurred before chest X-rays changes. The second group of animals presented later chest X-rays abnormalities and modifications of BAL components appeared at the same time or just before chest X-ray changes. These results should be confirmed on a larger population. Individual variation to dust exposure has also been described in humans. Koskinen et al. [43] have suggested that HLA groups may be implicated in this individual susceptibility but this hypothesis was not confirmed by other authors.

Our experimental model may be interesting since it allows sequential bronchoalveolar lavages and since monkeys possess strong phylogenetic relationship with humans. Among the factors involved in the destruction of lung parenchyma, we have demonstrated an increase in the release of superoxide anion by bronchoalveolar cells and an increase of glycosidases in BAL fluid. We have also demonstrated the appearance of newly arrived young macrophages which exhibit enhanced release of superoxide anion and, thus, may have an important role in the destruction of lung parenchyma. On the contrary, it was not possible to demonstrate a large increase of protease activity. Furthermore, inhalation of silica induces an increase of NEIC, which is implicated in lung defence.

The increase of glycosidases, and superoxide anion release may not be specific for silicosis but these factors are implicated in lung destruction. Furthermore, these parameters are also increased in BAL of subjects with well-established occupational lung diseases [44-47]. In our study we have shown that modifications occurred prior to modifications of chest X-rays and might be considered as early markers of this experimental model of silicosis.

References


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Essai d'évaluation de l'agression pulmonaire au cours de la silicose du singe a activité de péroxydase, de d'antiprotéases, et d'hydrolyases, dans des lavages bronchoalvéolaires répétés. M.H. Hannothiaux, A. Scharfman, A. Wastiaux, L. Cornu, E. van Brussel, J.J. Lafitte, P. Sebastien, P. Roussel. RÉSUMÉ: L'exposition à la silice peut induire des lésions de fibrose et/ou d'emphysème. Différents facteurs tels que les protéases, d'autres hydrolyases et les oxydants peuvent-être impliqués dans la dégradation du parenchyme pulmonaire. D'autre part les antiprotéases jouent un rôle important dans la protection du poumon vis à vis de l'action des protéases. Nous avons mis au point un modèle de silicose expérimentale chez le singe Macacus cynomolgus, et nous avons suivi ces facteurs par lavage bronchoalvéolaire (LBA). Nous avons étudié les glycosidases, une activité de type élastase, l'α-antiprotéase (αPI), la capacité inhibitrice vis à vis de l'élastase leucocytaire (C.I.E.L) et la myéoperoxidase. Une étude des populations cellulaires a également été réalisée. Un animal témoin est soumis à des lavages répétés. Six singes ont été exposés à des aérosols de quartz (100 mg·M· 3 ) pendant 18 semaines. Ils ont été suivis jusqu'à l'apparition d'anomalies radiologiques, qui sont détectées entre 21 et 64 semaines après l'arrêt de l'exposition au quartz. Des nodules silicotics ont été mis en évidence dans les biopsies pulmonaires. Des modifications des populations cellulaires ont été observées. La sécrétion d'anion superoxide par les cellules bronchoalvéolaires obtenues pendant l'expérience est augmentée. Après séparation sur gradient de Percoll, une population de macrophages jeunes est mise en évidence. Ces cellules ont une sécrétion d'anion superoxide accrue par rapport à la population cellulaire totale. L'analyse biochimique des LBA obtenus pendant et après arrêt de l'empoussièrement montre une augmentation des glycosidases, de l'αPI et de la C.I.E.L. Simultanément une activité de type élastase est détectée dans les LBA obtenus pendant et après empoussièrement, mais pas dans les LBA témoins. Cette activité est très faible par rapport à la C.I.E.L. L'augmentation des activités enzymatiques et des antiprotéases est observée à des délais variables selon les animaux suggérant des différences individuelles dans la réponse à la poussière. Ces modifications apparaissent avant les anomalies radiologiques.