Semi-quantitative X-ray microanalysis of bronchoalveolar lavage samples from silica-exposed and nonexposed subjects


ABSTRACT: To evaluate the possibility of quantifying alveolar dust burden in conditions of exposure to silica, four groups of subjects were submitted to bronchoalveolar lavage (BAL): 10 healthy control subjects and 39 patients affected by diffuse interstitial lung disease (DILD) never exposed to dust, 23 silicotic patients and 12 chronic bronchitis patients with a history of occupational exposure to silica dust. Five to ten million BAL recovered cells were analysed with an energy-dispersive X-ray microanalysis (EDXA) system to determine the silicon content, expressed in a semi-quantitative way as silicon to sulphur (Si/S) ratio.

The results were independent of smoking habit. The Si/S median values (interquartile range in brackets) for the four groups were 0.53 (0.5-5.65), 0.60 (0.41-0.8), 1.23 (1.6-1.39), 1.31 (1.11-1.97), respectively. Silicotics and simply exposed individuals did not show a significant discrepancy, but they were both significantly different in comparison with normal and DILD patients without history of exposure (p<0.001). 14.3% false negative cases were found, and 4.1% false positive cases (none among normal subjects). We did not see any significant relationships between the amount of silicon and the duration of exposure or the degree of chest X-ray involvement. A study of cytocentrifuge slides from the same subjects by polarizing light microscopy revealed a lower sensitivity (34% false negative cases).

From our study it can be concluded that: 1) it is possible to quantify the alveolar silicon burden on BAL samples, thus avoiding more invasive sampling techniques; 2) in our experimental conditions a Si/S level >0.95 (i.e. 95th centile value of all nonexposed subjects) is, in most cases, indicative of significant exposure to silica and/or silicates, compatible with pneumoconiosis but of no definitive diagnostic value, since nonsilicotic exposed subjects share the same values with silicotics; 3) a Si/S value <0.95 is typical of a nonexposure condition, but it may underestimate a marked dust inhalation in about 14% of cases.


The chronic inhalation of silicon containing minerals can cause a significant number of occupational lung diseases. Among these, silicosis is usually diagnosed on the basis of an exposure history, on the presence of typical chest X-ray findings and on the exclusion of other causes responsible for similar roentgenological pictures [1]. Sometimes, a chest X-ray compatible with silicosis is not supported by a history of definite and prolonged exposure to silica dust; alternatively, negative chest roentgenograms can be found in subjects with documented heavy exposure to silica dust and histological evidence of cryptic nodules [2].

In some cases, the presence of mineral particles can be detected by simple methods, which may help in making the diagnosis (e.g. identification and enumeration of asbestos bodies in sputum and alveolar lavage fluid) [3-6].

More sophisticated methods of qualitative and quantitative mineralogical analysis have been employed on an investigative basis, such as X-ray microdiffraction and electron-probe microanalysis on lung tissue samples [7-10], and, more recently, on bronchoalveolar lavage (BAL) specimens [11-14].

The simple detection of mineral particles is of no definitive value for diagnostic purposes, indicating exposure only, and, furthermore, such particles can be found in the general population [9]. Many attempts have, thus, been made to correlate disease (pathological and functional data) and risk of disease with quantitative mineralogical analysis data, particularly regarding asbestos body count in tissue and BAL fluids [3-6]. It has been shown that quantitative mineralogical analysis can give more interesting information than qualitative examination alone.
Different evidence has recently accumulated regarding the relationship between mineral fibre count in BAL and related pathology, but few attempts have been made to quantitatively measure the alveolar silica burden in cases of prevalent exposure to silica. CHRISTMAN et al. [12] determined the content of silica particles in alveolar macrophages obtained with BAL from workers not affected by silicosis, showing a significant increase of mineral dust in comparison with control nonexposed subjects.

In a preliminary study, we applied semi-quantitative energy-dispersive X-ray analysis (EDXA) to BAL samples, demonstrating a good specificity of the method in discriminating silicosis from diffuse interstitial lung diseases without history of silica exposure [13].

It has been demonstrated that the determination of the silicon level in lung biopsy samples by EDXA is useful in discriminating fibrosis due to silica exposure (silicosis) from other types of pulmonary fibrosis in silicotic subjects without histological evidence of silicosis [10].

The possibility of evaluating the silicon alveolar burden could be an important diagnostic aid in doubtful cases and where the patient history, roentgenological findings, or both, are inconclusive.

The mere presence of silicon does not represent a sufficient criterion for ascertaining a pneumoconiosis, since it simply indicates exposure, and this element can also be found in cells not directly in contact with the external environment, e.g. in red blood cells. The finding of silicon dioxide (SiO₂) as a normal blood constituent is well-known [15].

To evaluate the potential diagnostic usefulness of EDXA in measuring alveolar silicon levels, we compared data on subjects not exposed to silica dust with those from exposed patients, affected or not by silicosis. Comparison of EDXA with polarizing microscopy was also performed. Furthermore, the entity of silicon burden was correlated to the exposure data and the degree of chest X-ray involvement.

Materials and methods

Study populations

A total of 84 subjects was studied after giving informed consent. Table 1 reports some case history data and types of disease. Ten subjects were healthy controls (Group A), never exposed to silica dust. Thirty nine patients were hospitalized for a diffuse interstitial lung disease (DILD) (Group B); all had a negative case history for occupational exposure to silica dust. Diagnosis was achieved according to the standard clinical, laboratory, functional and histological criteria. They were selected from a group of 50 patients according to a rigorous case history survey, which excluded even occasional and suspected exposure. The remaining 35 experienced a heavy and prolonged occupational exposure to silica dust, but only 23 showed roentgenological (ILO classification) and functional signs of silicosis (Group C). Exposed patients without silicosis are referred to as Group D; they were admitted to hospital for chronic obstructive lung disease or suspected neoplasm. Table 2 (a and b) shows the main case history data concerning the exposure conditions.

Smoking was taken into particular account (table 1) since it induces marked differences in BAL features and in macrophage content of mineral particles [16].

Table 1. - Case history data for the 84 subjects, divided into smokers and nonsmokers

<table>
<thead>
<tr>
<th>Group</th>
<th>Nonsmokers</th>
<th>Smokers</th>
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<tr>
<td></td>
<td>n M/F</td>
<td>Age* yrs</td>
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<tr>
<td>A. Healthy controls</td>
<td>4 3/1</td>
<td>48±15 (36-68)</td>
</tr>
<tr>
<td>B. DILD**</td>
<td>18 9/9</td>
<td>49±18 (17-75)</td>
</tr>
<tr>
<td>C. Silicotics</td>
<td>12 12/0</td>
<td>60±10 (45-74)</td>
</tr>
<tr>
<td>D. Exposed nonsilicos</td>
<td>6 5/1</td>
<td>49±10 (50-58)</td>
</tr>
</tbody>
</table>

*: mean±so, with range in parenthesis; **: type of DILD and number of patients: Nonsmokers: sarcoidosis (8), hypersensitivity pneumonitis (6), idiopathic pulmonary fibrosis (4); Smokers: sarcoidosis (5), hypersensitivity pneumonitis (4), idiopathic pulmonary fibrosis (10), pulmonary alveolar proteinosis (1), histiocytosis X (1). DILD: diffuse interstitial lung disease.

Table 2. - Occupational data of the subjects exposed to silica

<table>
<thead>
<tr>
<th>Occupation</th>
<th>Years of exposure</th>
<th>Years since last exposure</th>
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Data are presented as mean±so, with range in parenthesis.

I: tunnel diggers; II: foundry workers; III: ceramic industry workers; IV: stone miller (glass industry).
Bronchoalveolar lavage

All subjects underwent BAL according to the procedure described previously [17]. Briefly, a fiberoptic bronchoscope was used to inject and then recover, with a syringe, three 50 ml boluses of sterile physiological saline solution into the medial segment bronchus of the median lobe or of the lingula. Recoveries were ≥40%. Fluids were collected in polypropylene plastic tubes and then filtered with a single layer mesh gauze.

Cell preparation

Cytocentrifuge (Cytospin 2, Shandon, London, UK) slides were prepared for studies by polarizing light microscopy after staining with May-Grunwald-Giemsa.

A sample of the recovered cells (5–10 million cells) was separated by centrifugation (800 ×g) and placed on pure carbon specimen mounts (Balzers Union Aktiengesellschaft, Balzers, Liechtenstein), which do not interfere with the analysis. After air-drying, the cells were completely dehydrated, firstly in a graded alcohol series and, subsequently, using a critical drying point technique in CO₂ environment (Critical Point Dryer, CPD 020, Balzers Union Aktiengesellschaft, Balzers, Liechtenstein). The sample was coated with a microscopic layer of conducting material (carbon) with a sputtering device (Carbon thread evaporation unit, CED 020, Balzers Union Aktiengesellschaft, Balzers, Liechtenstein).

Polarizing light microscopy

Two independent observers scored at least 200 macrophages per slide on at least two slides per subject to count the number of cytoplasmic birefringent particles by polarizing light microscopy at ×1,000 magnification. Data are expressed as number of particles per 100 macrophages.

X-ray microanalysis

X-ray microanalysis was performed with an EDAX-9100 system connected to a scanning electron microscope (SEM 500, Philips, Eindhoven, The Netherlands).

In this test, atoms from the sample are struck by an electron beam emitted from the filament of the SEM. The excited atoms emit photons at a wavelength dependent on the excitation energy level. The highest levels (innermost orbitals) emit X-photons at frequencies typical for each element. The X-photons are detected by an X-ray spectrometer, analysed, and recorded on the basis of their energy level, thus, obtaining an energy spectrum of emitted photons (figs 1 and 2). This profile is analysed by computer which calculates the element concentrations based on the peak areas as a percentage of the total scan area.

The test was performed at low magnification (×20) so that the electron beam impinges on the whole sample. This generates a value which represents the mean overall concentration of elements being measured. The various elements can have an extremely nonhomogeneous distribution at different points in the sample, but the mean value from a number of determinations performed at higher magnifications, e.g. ×100, and involving different parts of the sample closely approaches to the overall concentration detected with our procedure. Only semi-quantitative results are obtained. The quantity of elements (in atom % or weight %, the former being our choice for data expression) is reported in relation to sulphur. Origin of sulphur is from cells since all soluble sources (e.g. proteins) were discarded with supernatants after centrifugation. Indeed, comparison with reference standards having known concentration of silica or other elements is not possible because of the difficulties in reproducing definite characteristics of biological samples, such as thickness, surface features, and cellular density.

The procedure that we have adopted can avoid the cumbersome task of performing a microanalytical study on hundreds of single cells or particles to obtain reliable quantitative data.

Statistics

Values in tables are usually expressed as mean ±standard deviation. Since most data concerning silicotic and nonsilicotic silica-exposed patients are not normally distributed they are reported as median and range values (interquartile range for Si/S values in text, ranges being reported in figure 3). Kruskal-Wallis non-parametric analysis of variance was applied to compare all the different groups. The comparison between individual groups was made with Mann-Whitney U-test. Spearman's rank test or linear regression analysis as appropriate was used for correlation studies. A value of p<0.05 was considered statistically significant.

Analyses were performed using a microcomputer (Macintosh SE) and Stat-view + Graphics software (Abacus Concept).

Results

Polarizing light microscopy

Median number of refractile bright particles per 100 alveolar macrophages from healthy nonexposed controls was 13.5 (range 6–24), without significant differences between nonsmokers (8.5, 6–23.5) and smokers (16, 6.5–22.5, p=0.35). In DILD patients with no exposure, particle count (27, 3–68) was not significantly different from controls (p=0.26). Median particle count in all nonexposed subjects was 16 (3–68).
Microanalysis of BAL in Silica Exposure

Fig. 1. - An EDAX microanalytical spectrum from the BAL sample of a normal subject. The absolute prevalence of the sulphur (S) peak can be noticed. F.S.: full scale; EDAX: energy-dispersive X-ray analysis; BAL: bronchoalveolar lavage.

Fig. 2. - An EDAX microanalytical spectrum from a subject who underwent a heavy silica dust exposure. The silicon (Si) peak relevantly exceeds the sulphur (S) one. For abbreviations see legend to figure 1.

In silicotic and in exposed nonsilicotic patients particle values were 76 (23–149) and 65 (18–180), respectively, (p=0.49), and 75 (18–180) considering all exposed subjects together. Statistical comparison of the four groups was highly significant (p=0.0001), due to the "exposure" variable (both silicotic and simply exposed groups at least p=0.001 vs control and DILD groups).

Assuming the 95th percentile value of all nonexposed subjects (=62) as the upper limit of normal range we found 4.1% of false positive cases (limited to DILD group) and 34.3% of false negative cases, equally distributed in Groups C and D.

X-ray microanalysis

In normal healthy subjects the median silicon level as Si/S was found to be 0.55 (interquartile range 0.5–0.65) for nonsmokers, and 0.53 (0.45–0.65) for smokers (p=0.67). In DILD without history of silica exposure Si/S values were not significantly different in comparison with normal controls: 0.6 (0.4–0.8) for nonsmokers and 0.6 (0.5–0.7) for smokers (p=0.83). In healthy controls and DILD patients, i.e. in nonexposed
subject groups, results were, thus, comparable and they were not influenced by smoking or variations in cell differentials.

No significant discrepancies were found between nonsmoker and smoker subgroups in silicosis and simple exposure. Silicosis was associated with a higher Si/S, 1.3 (1.2-1.5) and 1.1 (0.96-1.21) for nonsmoker and smoker patients, respectively, (p=0.06), whilst Si/S levels in BAL samples from subjects exposed to silica dust but without clinical silicosis did not differ from those in silicotic patients: nonsmokers 1.52 (1.28-2.14), smokers 1.14 (1.1-1.8), p=0.34.

Furthermore, we considered the four groups on the basis of the smoking habits. The median values (interquartile range in brackets) obtained considering smoker and nonsmoker subjects together are 0.53 (0.5-0.65), 0.6 (0.41-0.8), 1.23 (1.06-1.39), 1.31 (1.11-1.97) for Groups A, B, C and D, respectively, as shown in figure 3, where 10th to 90th percentile range and individual values outside this range are also reported (Kruskal-Wallis test, p=0.0001). The latter two values are significantly different in comparison with both Group A, p<0.001, and Group B, p<0.0001 (Mann-Whitney U-test).

From our data it is reasonable to fix a Si/S=0.95 (i.e. 95th percentile value for all nonexposed subjects) as a borderline level, values above which mean a significant exposure to silica and/or silicates.

In this context, in Group A we did not find any false positive cases but two in Group B (5.1%), i.e. the method reveals quite a good specificity (4.1% false positive cases among all unexposed subjects). On the other hand, in Groups C and D there were 4 (17.4%) and 1 (8.3%) false negative cases, respectively. Considering the exposed patients all together the global percentage of false negative cases was 14.3%.

A simple regression analysis studying Si/S levels as a function of macrophage particle number (y=0.01x+0.66) showed a statistically significant F-test (p=0.015), but with rather low "r" (=0.48) and "r-squared" (=0.23) values.

False cases were not coincident between the two methods, the only exception being the false negative case with the lowest Si/S level (=0.27, 44 particles). In two exposed patients (one with silicosis) shown false negative by microanalysis, the particle count was positive. Taken together, the two methods show a global percentage of 2.9 false negative cases.

Detailed analysis of correlates between mineralogical and exposure data were performed only with values obtained by microanalysis, being the most sensitive of the two methods considered.

We did not find any significant positive correlations between years of exposure and Si/S levels, either in the single Groups (C and D) or in the global group of exposed subjects (p=0.2). It was not possible to quantify intensity of exposure objectively, thus, we were not able to evaluate the relationship between cumulative exposure and Si/S. Also, no correlation was found between years since last exposure and silicon in BAL cells (p=0.26). In silicotic patients the comparison between subjects with mild (p 1/1, p 1/2, 12 patients) and more prominent chest X-ray involvement (p 2/2, 11 patients) did not show significant differences in Si/S levels: 1.23 (interquartile range 1.1-1.58) and 1.22 (1.0-1.34), respectively.

The comparison between active workers with exposure at the time of BAL (eight subjects) and retired workers with more remote exposure (at least 2 yrs before, 22 patients) showed a higher median value in the nonretired group, i.e. 1.36 (1.16-1.92) vs 1.12 (1.0-1.4) but with a statistically significant difference (p=0.09).

Discussion

From a clinical point of view, diagnosis of simple silicosis is based mainly on a positive case history of exposure to silica dust and on a chest X-ray showing typical nodular opacities. That case history cannot define exposure accurately is a common possibility, particularly when exposure occurred many years before and compensation benefits are expected. In these cases high-resolution computed tomography (HRCT) and, eventually, biopsy can be recommended to correctly diagnose the type of fibrosis; analysis of the specimen with polarized microscopy may be helpful. Since microscopic analysis is not specific, and does not allow discrimination of simple exposure from overt disease, some authors have investigated the silicon levels of lung tissue using EDXA. Interestingly, they have found that in simple exposure without silicosis the silicon content of tissue was similar to that seen in nonexposed subjects, whilst it was significantly higher in silicosis [10].

BAL is a relatively noninvasive method to sample alveolar lining fluid from a wide parenchymal area. Cells in BAL fluids are mainly represented by alveolar macrophages, which are the first defence barrier against foreign agents reaching the terminal airways. The mineralogical/elemental study of BAL macrophages may, thus, represent an optimal tool for documentation of exposure to inorganic exogenous pollutants, potentially more valuable on a clinical basis than environmental measurements.

In the present study, we have compared two different methods of mineral analysis, polarizing light microscopy and EDXA, performed on BAL samples to evaluate whether they may provide useful data for: 1) the documentation of a marked silica exposure; and 2) a possible diagnostic role in discriminating simple exposure from silicosis.

Regarding the first point, data from both methods are consistent with quite a good specificity in differentiating a marked silica inhalation from a nonexposure condition, but that birefringent particles cannot be identified chemically needs to be specified.

Therefore, a positive result (i.e. Si/S >0.95) is almost certainly associated with a marked exposure to silica and/or silicates. Conversely, the presence of 14% false negative cases in Groups C and D indicates only a fair
sensitivity for the method, and appropriate caution must be paid in drawing any conclusions on a single case basis. The study of Funahashi et al. [10] on lung tissue reports a comparable percentage of false negative cases (13%). Polarizing microscopy reveals an even lower sensitivity (34% of false negative cases). Regression analysis shows that Si/S levels are a function of the birefringent particle variable but with only a rough correlation. The association of the two methods can improve sensitivity up to 97%.

Smoking habits do not seem to influence the level of silica alveolar retention as assessed with our methods, even if the presence of silicates in alveolar macrophages from smokers is well-known [16].

With regard to the second issue, we did not find any specificity in discriminating silicon content of BAL cells in silicosis and in simple exposure. We are not able to state whether Group D patients were affected or not by (cryptic) histological silicosis without clinical and roentgenological involvement [2]. Interestingly, there were subjects without overt silicosis who showed high levels of alveolar silicon even after 20–30 yrs of retirement from their dusty workplace.

Biopsies were not available in our cases of silica-exposed subjects and, thus, we could not compare data from BAL with those from tissue. As stated previously, there is documented microanalytical data obtained from biopsies demonstrating a significant difference, and thus a diagnosis merit, of silicon levels between silicotic and silica-exposed nonsilicotic subjects [10]. If it was possible to confirm a discrepancy in the presence of high silicon levels in the BAL cells but not in the lung tissue in silica-exposed subjects without silicosis, this would have important pathogenetic relevance.

Different patterns of particle clearance could be a possible explanation, but direct demonstration needs to be substantiated. A component of the dust clearance mechanisms consists in the penetration of particles through the respiratory epithelium directly into the interstitium [18]. If we hypothesize a similar alveolar macrophage dust content, the apparent discrepancy between the results of Funahashi et al. [10] and our study could be due to a different content of silicon in the interstitial tissue and, thus, to basic differences in clearance between exposed subjects who are prone to develop pneumoconiosis and those with low susceptibility.

In conclusion, according to our data a significant increase of silicon at the alveolar level is associated with a marked exposure in most cases. Care must be taken with diagnostic implications of exposure data when occupational case history may refer to inhalation of amorphous silica or silicates, such as talc and kaolin, which have less fibrogenic properties than quartz. For example, this could be the case for ceramic industry workers.

Therefore, in a patient presenting with a DILD a finding of a high silicon level in BAL cells cannot diagnose silicosis but it well defines a degree of exposure which may be associated with pneumoconiosis. In the same situation, but with a normal level of Si/S, the diagnosis of silicosis is unlikely, with a degree of uncertainty of about one case out of seven.

References