Analytical electron microscopy in pneumoconiosis[1]

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The study of pneumoconiosis has been greatly facilitated by use of the analytical electron microscope. Morphologic, crystallographic, and chemical analyses may be obtained on submicroscopic particles using various analytical modalities. Since there is no destruction of tissue, as in other analytical techniques, analytical electron microscopy can help relate the effects of foreign particles to localized cell and tissue reactions. An overview of the different modalities of the analytical electron microscope is presented, along with cases illustrative of their use.

Index terms: Microscopy, electron • Pneumoconiosis

Cleve Clin Q 52:503–512, Winter 1985

Inhalation of atmospheric particles and their accumulation in airways and alveoli has been associated with various pneumoconioses (silicosis, asbestosis, coal workers' pneumoconiosis, berylliosis, talcosis). The diagnosis of pneumoconiosis may be suggested by occupation, history, chest radiography, and routine light microscopy, but a multidisciplinary approach including electron microscopy plus analytical and quantification techniques is usually necessary to identify inhaled particles and study particle localization and exposure levels.

The identification of foreign substances in lung tissue may require dark-field microscopy, polarization microscopy, histochemical staining, and routine light and electron microscopy. But, while these techniques may be helpful in characterizing such substances, they do not always allow for precise identification of the foreign material. Other

[1] Department of Pathology, The Cleveland Clinic Foundation. Submitted for publication May 1985; accepted May 1985. ht

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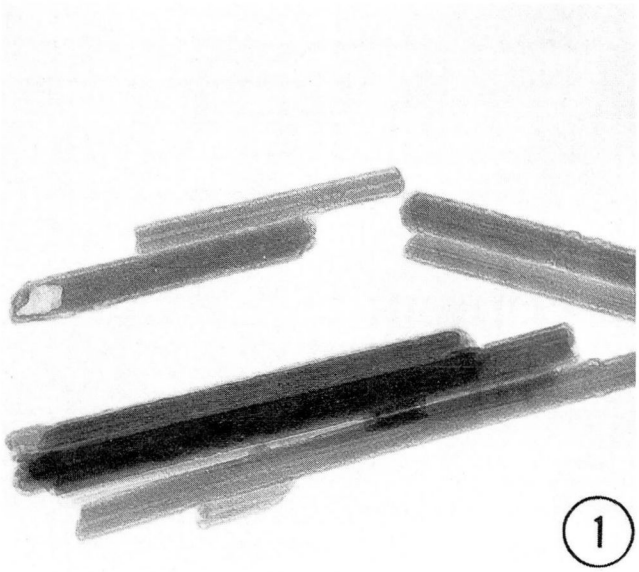

Fig. 1. Electron micrograph of chrysotile fibers. Electron-lucent cores occupy central portion of fibers and are distinctive features of chrysotile species of asbestos. (× 115,000)

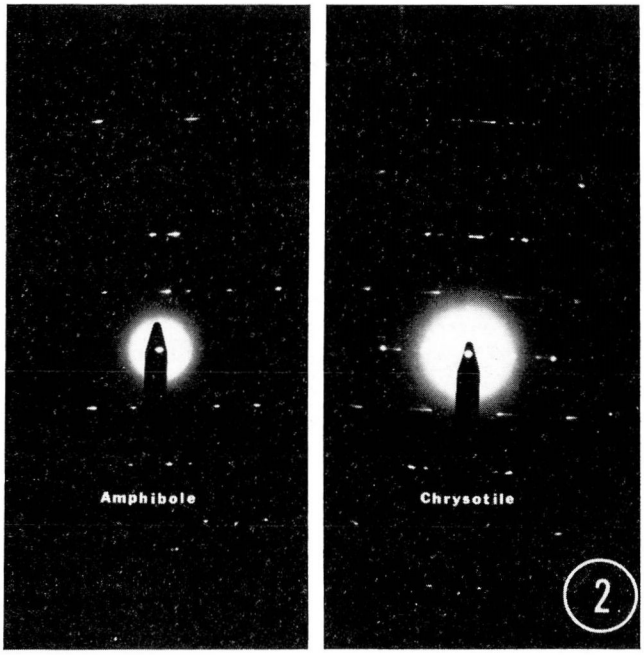

Fig. 2. Comparison of electron diffraction patterns of amphibole (left) and chrysotile (right) species of asbestos. Diffraction spots of amphibole are sharp and discrete. Diffraction spots of chrysotile appear streaked or blurred.

methods, such as microincineration and bulk chemical analysis, provide precise elemental identification, but require relatively large amounts of tissue and involve tissue destruction, thereby precluding any correlation between the disease process and the location and concentration of foreign particles.

Analytical electron microscopy (AEM) is a relatively recent development of ultrastructural techniques employing standard electron microscopes specially fitted with analytical equipment that allows nondestructive structural and *in situ* elemental microanalysis of lung and other tissues containing particulate materials.

AEM uses various emission signals that are produced as an electron beam is focused on a specimen. These emissions include transmitted and diffracted electrons that pass through the specimen and carry structural and crystallographic information, and signals that are emitted from specimen surfaces as either secondary or backscattered electrons carrying topology and density information. Interaction of the beam and the specimen also produces x-ray emissions that are characteristic of the molecules being irradiated. AEM incorporates a variety of detectors that can collect and use these signals and emissions in the identification and characterization of inorganic particles that may be of etiologic importance in dust-related pulmonary diseases.

Transmission electron microscopy and scanning transmission electron microscopy

As the beam generated in an electron microscope passes through the specimen, electrons are either deflected or absorbed in proportion to the density of the specimen and the reciprocal of the instrument accelerating voltage. Electrons that have penetrated the specimen are sensed on a phosphorescent screen in the case of transmission electron microscopy (TEM) or on a cathode ray tube (CRT) in the case of scanning transmission electron microscopy (STEM). Biologic structures visualized by TEM and STEM usually require augmentation of their electron density through the application of electron-opaque stains (osmium tetroxide, uranyl acetate, and lead citrate), while inorganic particles are frequently dense enough to be seen with no further enhancement. Examination of unstained material may allow for rapid

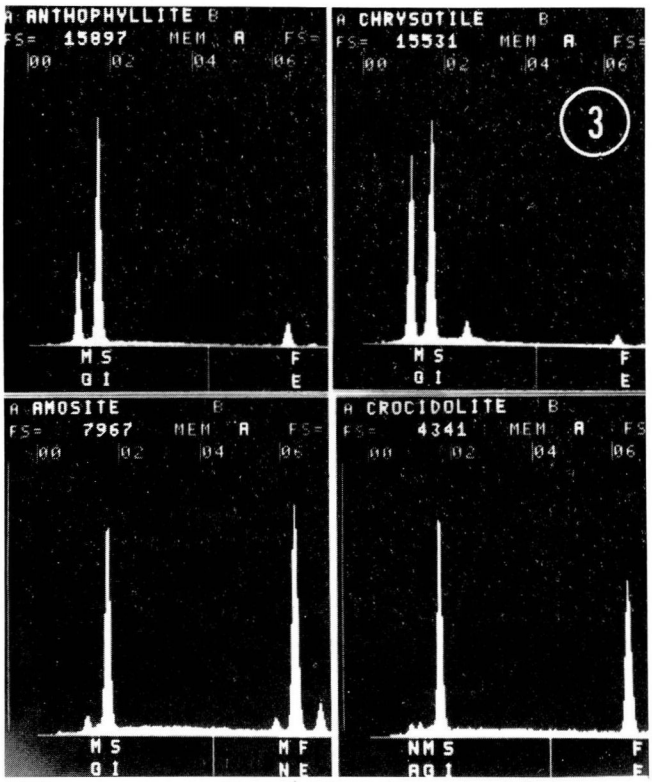

Fig. 3. X-ray spectra of each of the commercially important asbestos fibers anthophyllite, chrysotile, amosite, and crocidolite. Fibers differ in relative concentrations of magnesium (Mg), silicon (Si), iron (Fe), manganese (Mn), and sodium (Na) as demonstrated by height of respective spectral peaks.

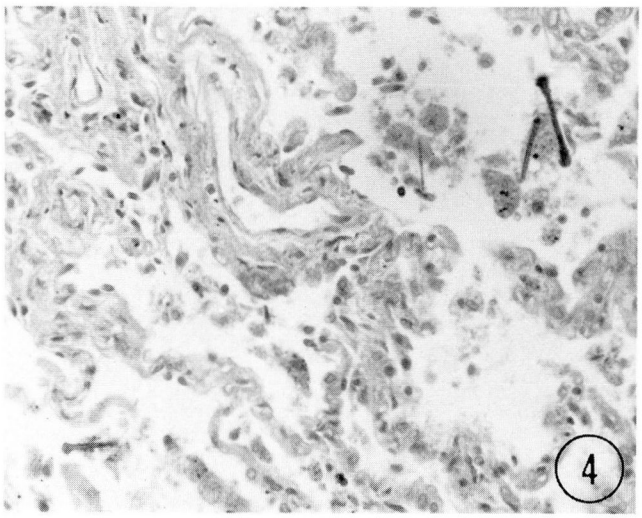

Fig. 4. Case 1. Micrograph of lung tissue from 36-year-old man having 27 months of heavy exposure to asbestos fibers. Ferruginous (asbestos) bodies are found within alveolar macrophages. (H & E, × 150)

location of particulate material contained within biologic sections and may permit subsequent elemental and crystallographic characterization of the particle without the encumbrances of stain interference. Visualization of relationships of particles to localized tissue reaction, however, may require the application of conventional electron microscope stains; but further analyses must distinguish stain contamination from particle chemical composition.

In most cases studied by TEM or STEM it is impossible to make particle identification based on morphology alone. For asbestos, however, chrysotile is the only fiber that has a central electron-lucent core (*Fig. 1*) that may be a significant ultrastructural observation when separation of asbestos fiber types is necessary.

The major advantage of performing TEM or STEM on lung specimens suspected of containing foreign particulate matter is the ability to detect and localize particles below the resolution of the light microscope and to establish their relationship to localized cellular responses.

Scanning electron microscopy

Scanning electron microscopy (SEM) using secondary electron imaging (SEI) has been widely used in the study of pneumoconioses since it allows topologic examination of large fields at magnifications of 20–100,000 ×, with identification and shape determination of particles too small to be seen by light microscopy. While SEI provides excellent visualization of particulate matter in tissue cavities, particles within intracellular spaces and dense stromal regions are more obscure, as are particles lacking distinctive shapes. The various modes of specimen imaging in the SEM, however, greatly increase the efficiency by which small or obscured particles are identified. Backscatter electron imaging (BEI) in the SEM provides an enhanced method of visualizing mineral particles in lung or other tissue, since it effectively contrasts specimen areas of differing average atomic density due to the greater backscattering of electrons by elements of high atomic number.[1,2] Foreign particles having an atomic density greater than the low-density lung parenchyma may be visualized as high-intensity areas that are usually distinguishable

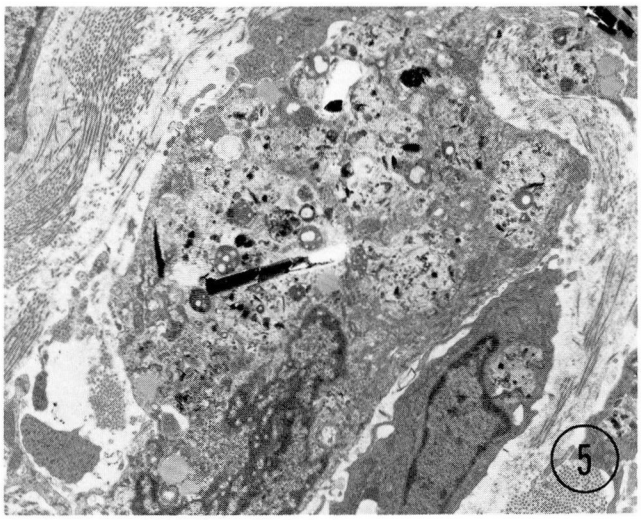

Fig. 5. Case 1. Electron micrograph of interstitial lung macrophage containing uncoated fibers of asbestos. X-ray and diffraction analysis indicated elemental and crystallographic composition consistent with that of amosite. (× 5700)

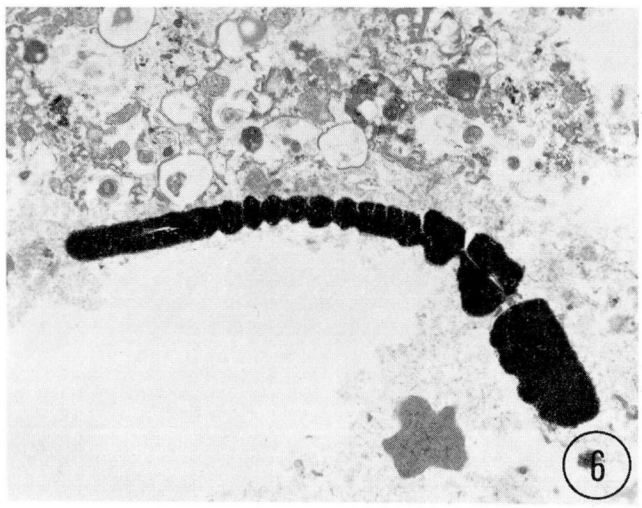

Fig. 6. Case 1. Electron micrograph of intra-alveolar ferruginous (asbestos) body. X-ray spectrum and electron diffraction pattern from asbestos core indicated the presence of amosite. (uranyl acetate & lead citrate, × 5200)

from a monotonous, dull background. Using BEI, mineral inclusions as small as 50 nanometers can be resolved and readily differentiated from other small tissue and cellular particles that may be otherwise indistinguishable using SEI. Particles detected by BEI can then be further characterized *in situ* using other detectors and imaging modes of the analytical electron microscope.

Selected area electron diffraction

Selected area electron diffraction (SAED) is an electron microscopic imaging technique that permits sequential morphologic and crystallographic analysis of mineral particles found in thin-sectioned specimens or isolated from lung or other tissue digestates.[3–5] SAED refocuses electrons following their crystallographic scatter into optical diffraction patterns that can be related to the periodic spacing of the crystal being analyzed. This technique gives additional structural information about a mineral that may be only partially characterized by other analytical means. SAED patterns, however, are difficult to interpret, and may present an even greater dilemma to the investigator without much experience. In the case of asbestos identification, however, patterns are distinctive and provide easy distinction between the major asbestos groups, chrysotile and amphiboles (*Fig. 2*), although amphibole fiber speciation may require additional x-ray analytical techniques.

Energy dispersive x-ray analysis

Although most commonly used by material scientists, energy dispersive x-ray analysis (EDXA) is becoming a popular and useful tool in pathology laboratories performing state-of-the-art electron microscopy.[6–11] EDXA is an *in situ* nondestructive analytical technique for recording the presence of elements from fluorine (atomic number Z = 9) to uranium (Z = 92) within the biopsy or surgical specimen. Analysis can be performed on very small, even subcellular, particles by the bombardment of the specimen by a narrow beam (minimum diameter = 50 nanometers) of high-energy electrons that causes individual atoms within the sample to emit characteristic x-rays that can be recorded, analyzed, and related to the elements present within the specimen and their relative concentrations.

EDXA is capable of detecting elements having a percent composition of 0.1–0.3% in a particle having a minimal mass of 10^{-18} to 10^{-20} grams. As EDXA is not sensitive to elements of low atomic number (Z < 9), organic compounds and particles including polyvinyl chloride, beryllium, and carbon must be analyzed by other means, e.g., atomic absorption. Since it is a nondestruc-

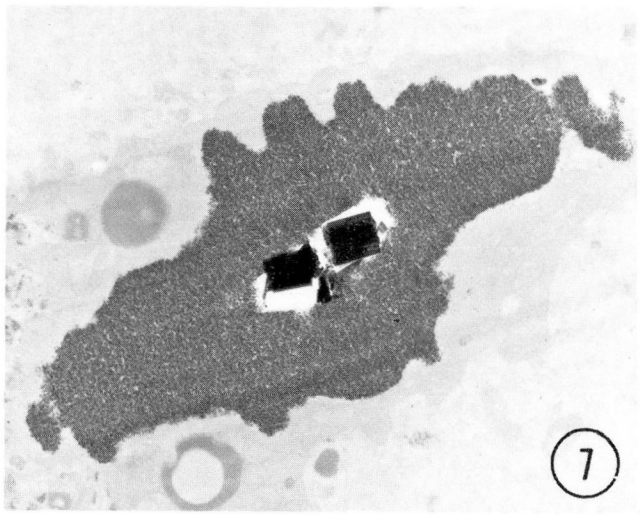

Fig. 7. Case 1. Electron micrograph of cross-section of ferruginous (asbestos) body within an intraalveolar macrophage. Note that central fiber does not contain electron-lucent core. X-ray and diffraction analysis indicated elemental and crystallographic composition consistent with that of amosite. (unstained, × 10,500)

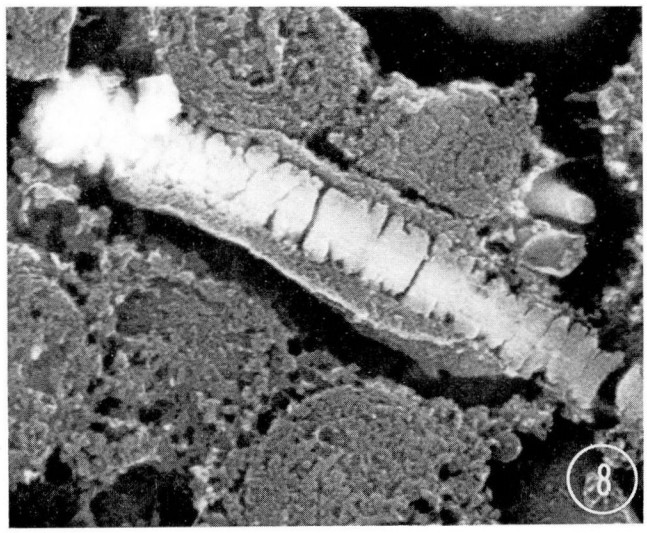

Fig. 8. Case 1. Scanning electron micrograph of ferruginous (asbestos) body within alveolar macrophage. Micrograph was taken from cut surface of deparaffinized histologic block. (gold coated, × 2000)

tive, *in situ* analysis, EDXA provides analytical information that can be correlated with the ultrastructural localization of particles. Combined with other analytical techniques, EDXA can also help point to distinctive physical and chemical properties of particles having fibrogenic or carcinogenic potentials. In asbestos-related diseases, EDXA produces distinctive spectral patterns that may be easily quantified for the identification and chemical separation of the commercially important asbestos fibers (*Fig. 3*).

X-ray mapping

As an imaging technique complementary to EDXA, x-ray mapping gives a visual presentation of elements within a particle or within a lung field. X-rays that are generated at the specimen level in either SEM or STEM and sensed by an x-ray detector are processed by a single channel analyzer and depicted as a distribution of dots on the screen of a CRT. The dot patterns can then be correlated with morphologic features to determine the concentration-related distribution of elements within a particle or the distribution of particles having similar elemental composition within a tissue field. While the technique is generally of limited application because of low resolution, it may have application in identifying particles of similar or dissimilar composition in a tissue field having particles too numerous to analyze individually.

Quantification of lung particulate load

Even with precise chemical definition and crystallographic characterization, quantitative analysis is essential for complete documentation of the pulmonary particulate load. Quantification is usually performed on the mineral residues following low-temperature ashing[12] or sodium hypochlorite (bleach) digestion,[6,7,13,14] although the former involves particle fracturing and may give an inflated numerical estimate. Counts may be made at either the light microscopic or electron microscopic levels. Even when using light microscopic aids such as polarized light and phase contrast optics, small particles and fibers readily escape detection, but are easily identified by electron microscopy. In the quantification of asbestos bodies (ferruginous bodies with asbestos cores), sediments from bleach digestion or ashing of preweighed portions of lung are collected on membrane filters (nucleopore or millipore) that are then cleared and mounted on glass slides for light microscopic counting or dissolved onto electron microscope grids.[13,14] The density of particles on the filter can be varied by resuspen-

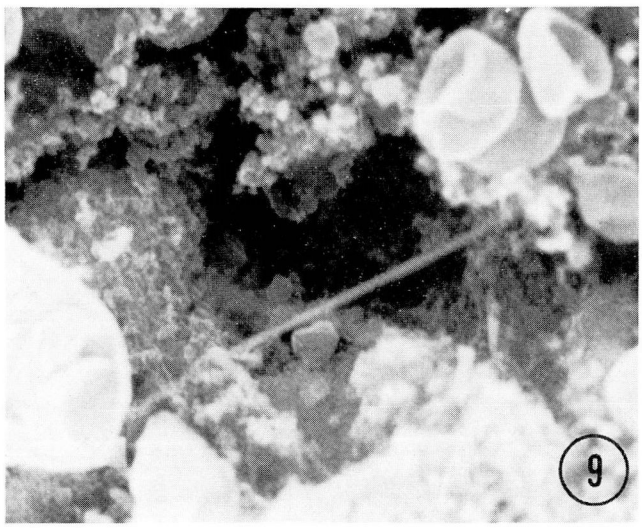

Fig. 9. Case 1. Scanning electron micrograph of nonsegmented asbestos fiber found within hemorrhagic alveolar space. (gold coated, × 2000)

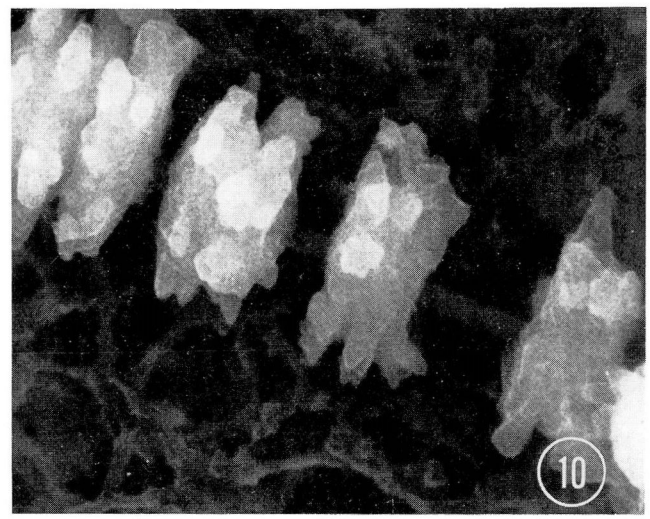

Fig. 10. Case 1. Scanning electron micrograph of ferruginous (asbestos) body isolated from lung digestate. X-ray analysis indicated elemental composition consistent with that of amosite. (gold coated, × 5000)

sion of centrifuged sediments in controlled diluent volumes of water and by aliquot sampling of the particle suspension. Quantification is achieved when particle counts are related to the original weight of the lung sample, the diluent volume in which the ashed or digested lung residue is suspended, and the aliquot sample that is counted. When analysis is performed on wet tissue, replicate specimens should be dried in order to obtain a dry weight equivalent. Analysis should be expressed in counts per gram dry lung.

In situ quantification is perhaps more useful, since it equates the local burden with regional differences in tissue response. *In situ* quantification may be performed by depositing 5-micrometer frozen or paraffin sections on carbon support mounts specially made for AEM, followed by removal of the paraffin substrate by solvent etching.[1] Calculated areas of the specimen are then scanned using BEI, which effectively differentiates atomic density, thereby providing clear contrast between the particulate matter and the low-density tissue parenchyma. The particles are then qualitatively identified using crystallographic or x-ray analytical methods, counted, and related to the volume of tissue analyzed. Since this technique requires less tissue and does not destroy the specimen, small specimens, even from needle biopsy, may be used. While the technique is time-consuming, it may provide identification and quantification of small particles below the resolution of light microscopy.

Procurement and processing of specimens

Characterization of particulate matter in lung and other tissues usually requires specimen fixation in solutions that do not interfere with crystallographic or elemental analysis. Formalin and glutaraldehyde fixations usually provide good structural morphology and do not interfere with chemical identification. Fixatives containing such metals as chromium, mercury, and zinc should be avoided if elemental analysis is indicated. Phosphate buffers and arsenic-containing cacodylate buffers may also present problems in analysis. EDXA of mineral substances in tissues post-fixed in osmium tetroxide for routine ultrastructural studies may produce overlapping elemental analysis (*e.g.*, phosphorus and osmium), but computerized subtraction of the osmium peaks may disclose the presence of anticipated or unsuspected elements. Retrospective studies may be performed on paraffin-embedded tissues, either by means of solvent etching of sections and BEI techniques already described, or by further processing and embedding for electron microscopy with analytical procedures performed on thin-sectioned material. In any case, specimens should be processed using safeguards so that no exogenous particles enter the tissue because of

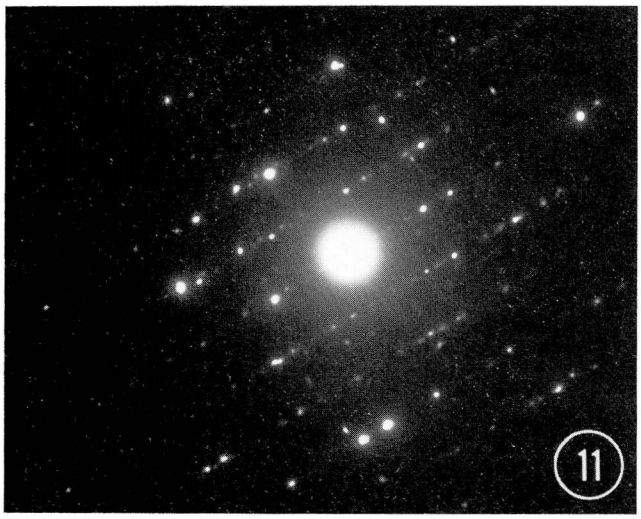

Fig. 11. Case 1. Electron diffraction pattern of asbestos fiber consistent with that of an amphibole. Compare with Figure 2.

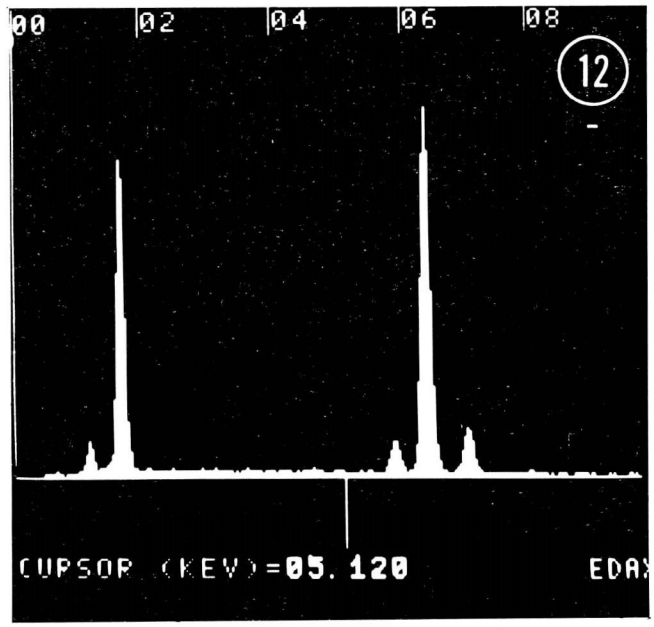

Fig. 12. Case 1. X-ray spectrum of amosite species of asbestos. Spectral peaks indicate presence of magnesium, silicon, manganese, and iron. Compare with Figure 3.

contaminated solutions. Filtration of fixatives, buffers, and alcohols before use usually prevents entry of contaminating particles into tissue spaces.

Illustrative cases

The cases presented here are intended to demonstrate the utility of the analytical electron microscope in investigating possible etiological factors in the development of environmentally related lung disease. In each case, there were supporting clinical and exposure histories along with radiographic evidence to suspect pneumoconioses.

Case 1. A 36-year-old man was employed 27 months, with heavy exposure to raw asbestos fibers, making casting molds for steel mills. The patient complained of weakness and malaise. Pulmonary function tests revealed decreased diffusion capacity and restrictive disease with resting hypoxemia. He had a small left pleural effusion. His chest radiographs showed a fine ground-glass interstitial pattern in the lower two-thirds of both lung fields.

Histologic sections (*Fig. 4*) of open-lung biopsy specimens showed diffuse interstitial fibrosis with ferruginous bodies identifiable as free within alveolar spaces and within alveolar macrophages. A combination of exposure history, radiographic findings, and histologic confirmation of lung fibrosis in the presence of asbestos fibers established the diagnosis of asbestosis, but did not indicate the type of asbestos present or other particles that may have contributed to the development of interstitial lung disease. Analytical electron microscopy was, therefore, indicated to identify the specific type of asbestos fiber present, and to identify any other particle that may have accounted for the progression of the patient's lung disease.

Asbestos bodies and fibers were identified by light microscopy (*Fig. 4*), TEM (*Fig. 5–7*), and by SEM using SEI of routine tissue preparations (*Figs. 8* and *9*) and lung digestates (*Fig. 10*). Quantification of asbestos-cored ferruginous bodies, using hypochlorite digestion and phase contrast optical counting techniques, set the lung particulate burden at 8.7×10^6 bodies per gram of dry lung. This value fell well within the range reported for asbestos workers having clinical and histologic asbestosis.[13] SAED of uncoated fibers and crystalline cores of coated fibers produced diffraction patterns typical of the amphibole class of asbestos fiber (*Fig. 11*). Further speciation of the fibers was achieved by EDXA and computer analysis of elemental concentrations. Peak ratio study of the spectral analysis (*Fig. 12*) indicated magnesium, silicon, and iron concentrations consistent with the amosite species of asbestos, while the significant presence of manganese supported this identification.[10] Numerous analyses consistently demonstrated similar spectra and indicated a heavy exposure to a single asbestos fiber type. Other regions of the lung specimen contained high concentrations of nonfilamentous electron-dense inclusions found within intra-alveolar spaces and within tissue macrophages (*Fig. 13*). Some of the particles were identified as quartz or free silica because of the EDXA-generated pure elemental silicon peak. Other particles were silicates that had analyzed compositions of aluminum, potassium, and silicon consistent with the mineral mica. In this case, AEM proved useful in identifying amosite as a major etiological component in the development of the patient's interstitial lung disease, with silica and silicates as potential cofibrogenic contaminants.

Case 2. A 55-year-old man was a farmer who also had a full-time job as a line worker in an ammunition factory where powdered mica was used to insulate live rounds from friction and possible spark production. The patient had been a nonsmoker for 20 years but had a previous 16-year history of smoking ½ pack per day. Upon admission, the patient

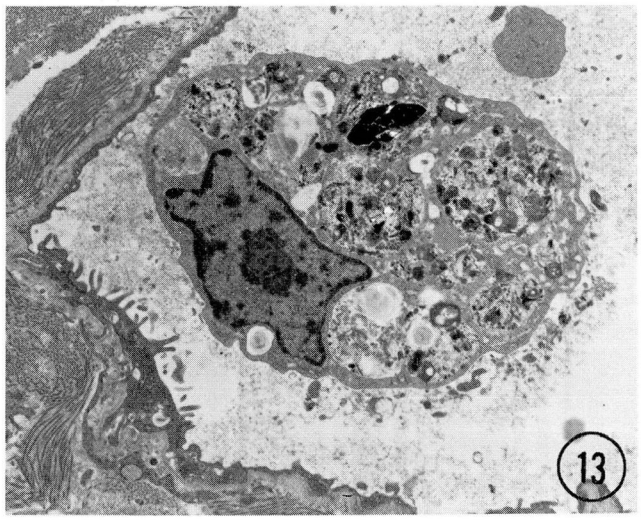

Fig. 13. Case 1. Electron micrograph of intra-alveolar macrophage containing large phagocytic vacuoles. Within one vacuole is an electron-dense particle with x-ray analysis indicating a composition of aluminum, potassium, and silicon (mica). (uranyl acetate & lead citrate, × 4900)

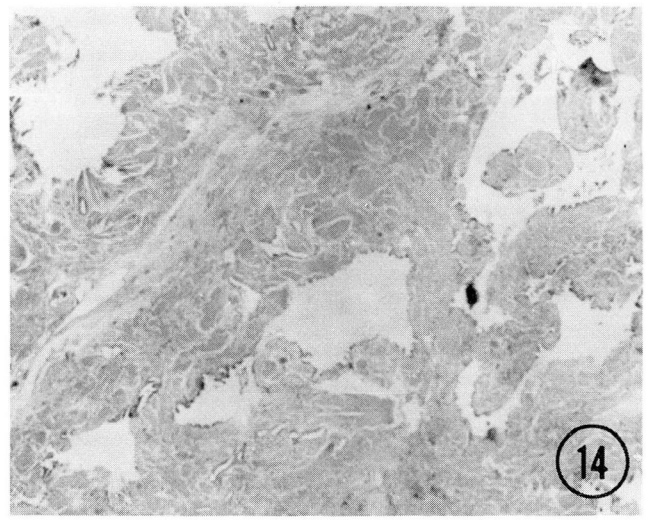

Fig. 14. Case 2. Micrograph of lung tissue from 55-year-old man having history of exposure to powdered mica. There is severe interstitial fibrosis with obliteration of normal lung architecture. (H & E, ×150)

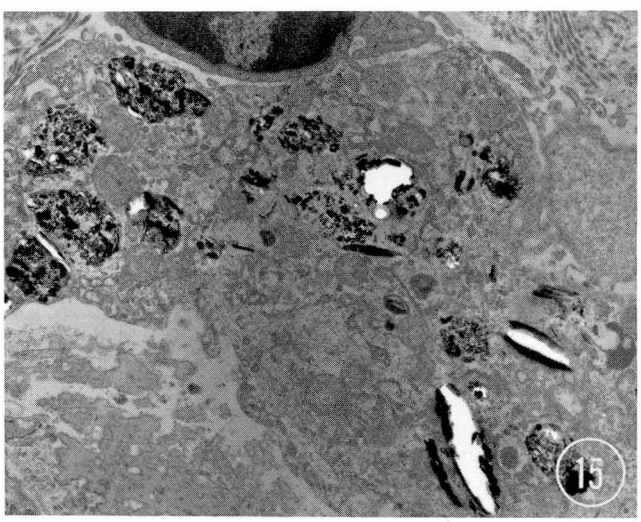

Fig. 15. Case 2. Electron micrograph of pulmonary macrophage containing numerous electron-dense particles having x-ray analyses consistent with the presence of aluminum potassium silicates (mica). (uranyl acetate & lead citrate, × 8700)

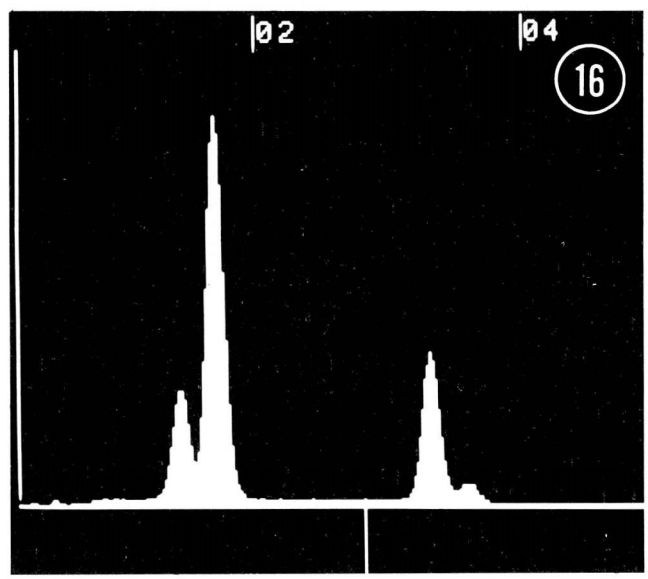

Fig. 16. Case 2. X-ray spectrum of mica. Spectral peaks indicate presence of aluminium, silicon, and potassium.

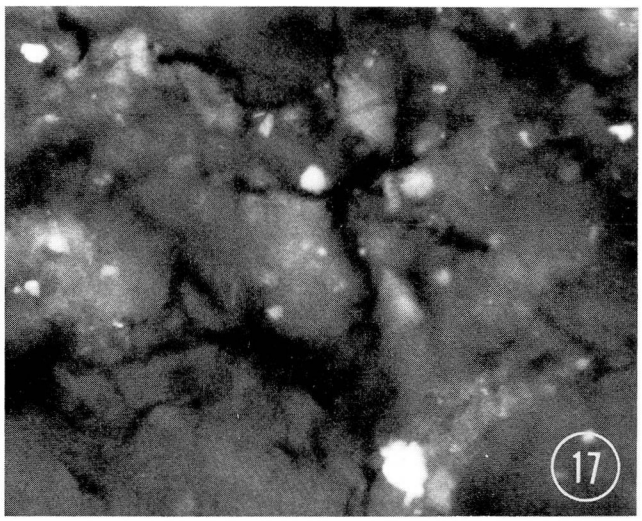

Fig. 17. Case 2. Backscatter electron image (BEI) of fibrotic lung field. Numerous small particles are sharply identified against a dull monotonous stroma. (× 2200).

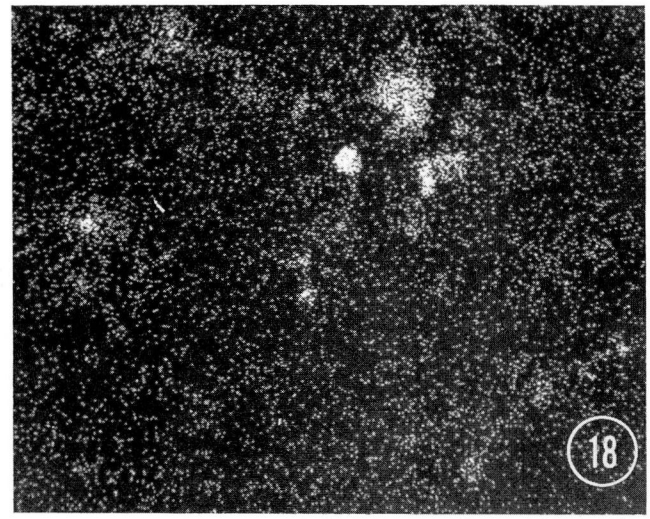

Fig. 18. Case 2. X-ray dot map corresponding to Figure 17. Particles containing silicon produce concentrated clusters of white dots. Particles not containing silicon should be analyzed separately using energy dispersive x-ray analysis.

complained of exertional dyspnea with physical examination showing clubbing of nails, and inspiratory fine crackles in both lung bases. Chest radiographs showed extensive reticular interstitial markings involving both lung fields, especially the bases.

Histologic sections demonstrated a honeycomb lung with severe interstitial fibrosis and a mild inflammatory infiltrate (*Fig. 14*). Polarization microscopy demonstrated widely scattered, readily discernible particles, and a fine distribution of particles of sizes near the resolution limit of light microscopy. TEM of a routinely embedded lung specimen revealed numerous macrophagic cells containing cytoplasmic lysosomes having electron-dense, angulated mineral contents (*Fig. 15*). EDXA of these inclusions generated spectral peaks consistent with the silicon, aluminum, and potassium content of mica (*Fig. 16*). Lung specimens submitted for low-temperature ashing and chemical analysis also demonstrated the presence of aluminum potassium silicates.

In an attempt to study the distribution of small submicroscopic particles more critically, frozen and solvent-etched paraffin sections were examined in the SEM mode using BEI. Since the sections prepared for BEI were from the same blocks as those examined by light microscopy, the local effects of small-particle contaminants could be more closely related to fibrogenesis using the greater resolving power of SEM. In large fields studied by BEI (*Fig. 17*) and x-ray mapping (*Fig. 18*) at low magnification, there was a more intense distribution of particles than could be appreciated by bright-field or polarized-light microscopy. The particles appeared to be within a dense stromal matrix corresponding to fibrotic areas seen by light and electron microscopy. EDXA and dot mapping analysis of these particles reflected the findings using TEM/EDXA, although particles of other composition were also found.

AEM in this case provided a better correlation between small mica particle contamination and the possible fibrogenic reaction of tissue to the presence of those particles. In addition, AEM gave a better appreciation of the patient's exposure level to submicroscopic mica particles than could have been gained by light microscopic studies alone.

Conclusions

AEM is a useful approach to the study of environmental lung disease, since it permits the identification and characterization of inorganic dust in relation to specific tissue changes. Since identification may be made on particles too small to be seen by the light microscope, AEM may help affirm pathologic diagnoses in which exposures are suspected, but histologically undetected. AEM may also provide information on the pathologic potential of specific mineral particles and help establish tissue exposure levels compatible with disease. In the cases presented, diagnoses were made at the histologic level, but AEM helped establish positive correlation between specific lung contaminants and local cellular and tissue reactions.

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References

1. Abraham JL, Burnett BR. Quantitative analysis of inorganic particulate burden in situ in tissue sections. Scanning Electron Microsc 1983; **2**:681–696.
2. DeNee PB, Abraham JL. Backscattered electron imaging: Application of atomic number contrast. [In] Hayat MA, ed. Principles and Techniques of Scanning Electron Microscopy. New York, Van Nostrand Reinhold, 1976.
3. Churg A, Warnock ML. Analysis of the cores of ferruginous (asbestos) bodies from the general population. I. Patients with and without lung cancer. Lab Invest 1977; **37**:280–286.
4. Langer AM, Mackler AD, Pooley FD. Electron microscopical investigation of asbestos fibers. Environ Health Perspect 1974; **9**:63–80.
5. Beeston BEP, Horne RW, Markham R. Electron Diffraction and Optical Diffraction Techniques. New York, American Elsevier, 1973.
6. Lechene C. Electron probe microanalysis of biological soft tissues: Principle and technique. Fed Proc 1980; **39**:2871–2880.
7. Funahashi A, Schlueter DP, Pintar K, Siegesmund KA. Value of *in situ* elemental microanalysis in the histologic diagnosis of silicosis. Chest 1984; **85**:506–509.
8. Langer AM, Rubin IB, Selikoff IJ. Chemical characterization of asbestos body cores by electron microprobe analysis. J Histochem Cytochem 1972; **20**:723–734.
9. Hayashi H. Analytical electron microscopy in the study of pneumoconiosis. Environ Res 1982; **23**:410–421.
10. Rubin IA, Maggiore CJ. Elemental analysis of asbestos fibers by means of electron probe techniques. Environ Health Perspect 1974; **9**:81–94.
11. Sherwin RP, Barman ML, Abraham JL. Silicate pneumoconiosis of farm workers. Lab Invest 1979; **40**:476–582.
12. Thomas RS, Hollahan JR. Use of chemically-reactive gas plasmas in preparing specimens for scanning electron microscopy and electron probe microanalysis. Scanning Electron Microsc 1974; **1**:83–92.
13. Churg A. Fiber counting and analysis in the diagnosis of asbestos-related disease. Human Pathol 1982; **13**:381–392.
14. Churg A, Sakoda N, Warnock ML. A simple method for preparing ferruginous bodies for electron microscopic examination. Amer J Clin Pathol 1977; **68**:513–517.