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QUANTITATIVE ANALYSIS OF INORGANIC PARTICULATE BURDEN IN SITU IN TISSUE SECTIONS

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Abstract

Quantitative information on tissue particulate burden is essential for investigation of relationships of particulates to several aspects of disease, such as exposure, physiology, radiology and pathology. We have developed a method for quantitative analysis of inorganic particulate burden in situ in tissue sections using scanning electron microscopy (SEM) with backscattered electron (BSE) imaging and energy dispersive x-ray analysis (EDXA) or the ion microprobe mass analyzer (IMMA). Our data show reproducible results for concentrations above 10^6 particles per cm^3 of tissue. Samples of less than one microgram can be analyzed. Standard 5 μm paraffin sections of formalin fixed tissue are mounted on carbon discs, deparaffinized and examined in the SEM under standardized conditions. Fields of view comprising 10,000 μm^3 are searched and all particles visible in the BSE image are counted and individually analyzed by EDXA. Location, number, size and types of particles are tabulated. Previous work showed correlation of quantitative SEM/EDXA with bulk analysis for Ni (r=0.64), and IMMA with bulk analysis for Be (r=0.999). Analytical results on exogenous and endogenous particles in 40 lungs (>6000 fields searched and >5700 particles analyzed), and the pathology observed in the tissue, may serve as baseline for future studies on all types of inorganic particles in normal and abnormal lungs. Observed concentrations ranged from $3x10^6$ to $2x10^9$ exogenous particles per cm^3 of tissue.

KEY WORDS: Lung pathology, particulates, backscattered electrons, quantitative analytical methods, pneumoconiosis, energy dispersive x-ray analysis.

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Introduction

Inhalation of atmospheric particles may have several measurable effects. These include: 1) aggravation of existing respiratory and cardiovascular disease; 2) effects on respiratory mechanics and symptoms (e.g. cough); 3) effects on clearance and other host defense mechanisms; 4) morphological and cytological alterations; 5) carcinogenesis and 6) mortality (16).

Quantitation of pulmonary dust burden is necessary for several reasons. Testing association of particulates with reactions of the lung requires measurements sufficiently quantitative to determine correlation of tissue burden with exposure, physiologic, radiologic and pathologic data. Several questions are: What is the minimum sample necessary? What is the most efficient method? What detection limits can be expected? How do the results compare with independent analyses of the same tissue samples using other methods?

The particulate burden of the lung can be measured by tissue destruction (ashing, digestion) followed by bulk chemistry and/or isolated particle analysis. Why then is there any need for a method for quantitative in situ analysis of particulates in a single section of tissue? Bulk chemical analyses usually require approximately one gram of tissue. The associations of elements within particles (which may provide clues to sources of the particles) and particle size information are lost when bulk chemical methods are used. However, analysis of individual isolated particles avoids these problems. Any techniques destructive of tissue obviate the chance to correlate the cellular and tissue changes (i.e., pathology) with the types, locations and concentrations of particulates. Thus the two main indications for in situ quantitative tissue analysis for particulates are: 1) when correlation of pathologic reaction and particulates are of

interest and/or 2) when insufficient tissue is available for bulk analysis. This report presents the background on development of in situ quantitative analysis of inorganic particulates in the lung. The current methodology and resulting data from a varied group of 40 cases are presented.

Materials and Methods

Tissue sampling. The method is applicable to any tissue sample large enough to yield an area suitable for searching as described below. Open biopsies are preferable to closed (e.g. needle or transbronchial), as the latter do not allow sampling of all portions of the lung parenchyma. The question of whether the sample taken is representative of the lung as a whole cannot be resolved by definition of a minimum volume of tissue. The selection of the site(s) must be made by the surgeon (biopsy) or pathologist (autopsy) after careful review of the history, radiology and physiology. Good medical practice precludes a random lung biopsy site selection. Gross inspection and palpation of the lung should be made to aid selection of representative areas (18). At present the variation in particulate burden with disease has not been adequately described; more work is needed. However, it has been reported that there may be up to an order of magnitude variation in asbestos fiber counts from one region of the lung to another (29). The variability and validation of our method are discussed below.

Fixation of tissue. We prefer standard 4% formaldehyde (10% formalin) fixed tissues (0.1M phosphate buffer does not interfere with our analysis). However, the only restrictions are that the fixative used does not contaminate the tissue (e.g. Hg-containing Zenker's fixative, Zn-containing or Os fixative). It is not standard practice to filter fixatives in pathology laboratories. In analyzing particles recovered from tissues by destructive techniques such contamination is a potential problem. However, with our in situ method it is possible to exclude such contaminant particles which would not be intracellular.

Embedding, Sectioning and Mounting of Sections for SEM. Standard paraffin wax, which is easily removed from the mounted section, is the easiest to use embedding medium. Frozen sections may be used, as may plastic. When plastic is used, it should be easily removable and should not contaminate the tissue with extraneous elements. The smooth surface of sections of unremoved plastic hinder examination of morphology and make identification of particles for analysis difficult by raising the backscattered electron yield of the tissue and void spaces, and also by hampering the determination of whether a particle is on, in or under the tissue.

Standard section thickness is 5μm. This provides a maximal thickness for detection of particles in the section by BSE imaging and thereby allows maximal sampling of tissue volume (see ref. 1).

Paraffin sections are floated on a warm water bath and picked up onto smooth pure carbon discs in the same manner as onto routine glass slides for light microscopy. The sections are affixed to the discs by heating at 60°C for an hour or more. Then, after cooling to room temperature, the paraffin is removed by soaking in xylene for at least 30 minutes. The section is allowed to dry directly from xylene. There is no need for critical point drying to study inorganic particles.

Transfer of sections from glass slides to carbon. On rare occasions when no paraffin block is available, a previously prepared section (stained or unstained) can be used for quantitative in situ microanalysis. If stained, the stain must not interfere (e.g. silver stains). Some bromine can be detected from the eosin in hematoxylin and eosin (H and E) stained sections, and this may occasionally cause problems due to overlap of the Br EDXA peak (L line) with Al (K line). The actual transfer of the section involves: 1) removal of the cover slip and mounting medium (if present), 2) destaining (if necessary), 3) covering the section with a film of suitable polymer such as acrylic plastic, 4) allowing the film to harden, 5) removal of the section or desired portion of the section from the slide and transfer to the carbon disc, 6) affixing it to the disc with heat, and 7) removal of the medium.

Coating for conductivity is rarely needed for 5μm deparaffinized sections on carbon, if examined in the SEM with high kV beam (20kV). When initial examination in the SEM reveals charging, then coating with carbon is utilized. In no case is metal coating used, as it interferes with both BSE imaging and EDXA analysis.

Light Microscopy. All sections are first examined by light microscopy using routine H and E as well as elastic van Gieson stain for fibrosis and vascular and airway changes and iron stain for iron accumulation and asbestos (ferruginous) bodies. Brightfield and polarized light are routine. If excess acid hematin (formalin pigment) is noted to interfere with polarized light examination, hematin can be removed with saturated aqueous picric acid overnight treatment of the section. Neither the

formalin pigment nor the picric acid treatment interferes with SEM or EDXA analysis.

Quantitative Analysis. Our analyses are carried out on an ETEC Autoscan SEM equipped with a standard solid state BSE detector (6) and a computer based KEVEX system with a 30 mm^2 detector. Quantitative analysis of particles in the tissue are carried out under standardized conditions: 45 degrees tilt of specimen; 14 mm working distance; 20 kV accelerating potential, 1 nanoamp beam current at specimen and 3.5 cm specimen to x-ray detector distance. In the scanning electron microscope each section on carbon disc is photographed at 70X and a montage created (Fig. 1). Fields are searched at a magnification of 6000X on the viewing screen of the SEM which corresponded to an area of 2000 μm^2 and a volume of 10,000 μm^3. Each successive field is chosen by a small but random turn of the horizontal axis control of the SEM stage goniometer and the location of each field marked on the montage by a felt-tipped pen (Fig. 1B). The montage reference is useful for three reasons: 1) it provides a record of all the specific morphologic areas searched as well as those in which particles were found, 2) it conserves film and time by eliminating the necessity for separately photographing each field at high magnification, and 3) it allows return to any given field and particle if reanalysis becomes necessary in the future. Each field is examined both in secondary electron (SE) imaging and backscatter electron imaging for variations in electron scatter (i.e. for presence of inorganic particulates within the tissue). Particles on top of, under or away from tissue are not considered for analysis (Fig. 2).

For each field we record field number and number of particles. For each particle we record the location within the tissue; the size in one dimension (a spherical particle) or two dimensions (irregular or fibrous particle) and the net x-ray counts per second observed for each detected element during a twenty second analysis at high magnification (i.e. beam raster confined to the particle). The background spectrum from the supporting carbon disc is very stable and reproducible and is subtracted from each particle spectrum to yield a net spectrum.

Each case is analyzed by counting the number of particles in at least 100 consecutive random fields. If less than 100 particles are found in the first 100 fields an additional 25 fields were searched (or multiple thereof) or until the time allocation for that particular day expires on the SEM (after 100 particles determined). In order that we do not spend an inordinate amount of time on a single case, we analyze only up to 20 particles in a field. In any field containing >20 particles, the particles are all counted and the compositional results of the first 20 (randomly analyzed) are extrapolated to the entire number of particles from that field.

The average case requires approximately 5 hours of microscope time plus a variable amount of time for data tabulation and analysis. The major factors at present precluding automated analysis are those decisions required by the operator: 1) interpreting the morphology of the tissue section, 2) deciding whether a particle seen in the BSE image is in, on or under the tissue, 3) determining the particle shape for particles needing stereometry and 4) resolving overlapping or aggregated particles.

The EDXA data allow separation of particles into two major classes: endogenous and exogenous. Particles containing Ca and P, or Fe and P as major constituents with sometimes smaller amounts of Na, Mg, and K are considered endogenous, and the remaining particles are considered exogenous. Exogenous particles are divided into three major classes: silica (showing only a silicon peak); silicates (showing silicon plus other cations; e.g. Al, Mg, K, Ca, Fe); and other (mostly metals; either singly or in combinations).

Special Problems

Contamination by fixatives is discussed above. Each lot of carbon substrate discs should be screened by BSE imaging to avoid occasional lots contaminated or polished with small particles (e.g. silicon monoxide or alumina abrasives). Endogenous vs exogenous particles have been defined above. Heavily calcified tissue sections can be treated with dilute (1%) acetic acid to decalcify them. Tissues with large endogenous iron (hemosiderin) burdens present a currently insurmountable problem. If one must analyze 100 or 1000 endogenous particles to find one exogenous the analysis becomes too inefficient. We have yet to find a way to remove endogenous iron which might not also destroy exogenous particles.

Since respirable particles in the lung are generally <5μm, the analyst should be suspicious of larger exogenous particles. However, larger particles can reach the lung during life by intravenous injection (e.g. in drug addicts) or by aspiration of material intended for the gastrointestinal tract (4).

Aggregations and overlap of particles are a problem in some cases, as it is even with particles isolated from tissues. The detection of an aggre-

gate or overlap depends first of all on the spatial resolution (dealt with below). Once detected, the appearance of the individual particles in the BSE image is useful in evaluating whether the aggregate is a mixed group (as might occur in a lysosomal package in a macrophage after phagocytosis of several separate particles) or uniform (as in some welding fume aggregates). Analysis of several regions within the aggregate is performed to see if there are any differences in the types and/or proportions of different elements. Aggregates which cannot be resolved into separate component particles by analysis are counted as one particle which is categorized as a separate type. Superimposed particles can be frequently deconvoluted morphologically by noting, e.g. a tiny particle with higher BSE signal on top of a larger one with lower BSE signal. The analysis of the smaller one will show the composition of both, but by analyzing the underlying one separately (as far as possible from the higher BSE particle) its spectrum can be stripped from the combined one resulting from the analysis of the smaller one. This approach, combining specimen topography, BSE yield and EDXA data, allows us to deal with most aggregates and overlaps. These few which cannot be separated are counted as single particles with mixed composition.

Fibers present special problems for in situ analysis. These are the particles which have received most attention. Most quantitative reports deal with fibers only and do not cover the large numbers of non- fibrous particles in the lung (32). Our method of analysis classifies particles as fibers ("possible fibers") if they show no evidence of platy structure in either secondary or BSE images and they have parallel sides and an aspect ratio of at least 4 to 1 (length to diameter). We recognize that some platy particles on edge may be misclassified as fibers, and that fibers oriented in certain directions (e.g. perpendicular to the plane of tissue section) will appear to be round or irregular rather than fibrous. Without destroying the tissue this is the best that can be done. If fibers are too small to resolve or present at too low a concentration in the tissue, their presence may also go unnoticed. In our experience based on analysis of a group of cases with known fiber exposure (see Validation and Results sections, below) we feel that significant fiber exposures will not be missed with our method.

Low atomic number particles even if detected with BSE imaging will yield no identifying data from EDXA spectra. Thus, a significant particulate exposure to organic or low atomic number (Z) materials may not be revealed using SEM/EDXA. Major exposures to opaque low Z particulates such as coal or graphite will, however, be revealed by light microscopy, even if analysis is not done by SEM/EDXA. For those cases requiring special analysis of particles containing light elements (e.g., Be), the IMMA can be utilized, either qualitatively or quantitatively (2). Organic and molecular identification of particles visible by light microscopy can be accomplished using laser Raman microprobe analysis (7,11).

Precise mineralogic identification of particles is not usually provided by SEM/EDXA. The groupings of particles into chemically and morphologically similar clusters is adequate for initial assessment and for comparison with similarly analyzed environmental samples (1). Single particle crystallography requires other techniques such as TEM with selected area electron diffraction or laser Raman spectroscopy.

Ultrastructural, subcellular morphologic detail is not usefully recognizable with SEM of paraffin sections. In the case where ultrastructural studies of particle type are necessary, one must sacrifice quantitative analysis and utilize TEM or STEM of stained thin sections.

Testing of the Method

Limits of detection

For quantitative studies, there are three limiting factors in our method. These are: 1) the number of fields examined; 2) the size and atomic number of the particle (SEM resolution and BSE contrast); 3) the sensitivity of the EDXA system. If only one particle of any given type per 100 fields is found, this would correspond to a concentration of 1.0×10^6 particles/cm^3 (0.5×10^6/cm^3 if 200 fields are searched, etc.). It is worth emphasizing that the sampling volume is a major limiting factor in analysis of particles in sections. TEM or STEM analysis of thinner sections requires approximately an order of magnitude more time to survey the same volume of tissue. In practice TEM or STEM is inefficient for quantitative in situ analysis since the concentration range found in some diseased tissues and many normals is in the range of 10^6 - 10^7 particles/cm^3. This range is within the detection limits in our method. The magnification and BSE detector we use can detect particles as small as 0.05 μm in diameter. This varies somewhat with atomic number, but includes most respirable particles. It has been estimated that 80% of lung particles (fibers) are greater than 0.05 μm diameter (27). The limits of detection for an element in a

detected particle during our 20 sec analysis is approximately 0.1% by weight. (e.g. 10 counts/sec under conditions yielding 10,000 counts/sec for a pure element standard).

Variability

Several aspects of our method have been tested in addition to the data presented with specific cases in the Results section, below. These are presented in Table 1.

Repeated analyses of the same section by the same person gave the results shown in Table 1 for cases 12,13 and 8. The rather large standard error of the mean results largely from the uneven distribution of particles in the section.

Analysis of the same section by different analysts in cases 34 and 10 showed no significant variation ($p>0.05$). The second analyst had only one month training and experience before these analyses were done. The effects of decalcification on analysis by the same analyst are shown for case 8, and for the different analysts for case 10 (in Table 1). The elimination of a large number of endogenous calcium-containing particles generally decreases the BSE background and may increase the sensitivity for exogenous particles. Also, the removal of large numbers of endogenous particles increases the efficiency of the analysis for exogenous particles. The final concentration of exogenous particles should be, and is, unchanged.

The variation with different blocks (different pieces of tissue) from the same lung is shown in cases 35, 36 and 18. Case 36 compares an inflated and uninflated section of the same nearly normal lung, and in case 18 serial sections from the same three blocks of tissue are examined. There is more variation from one lobe of the lung to

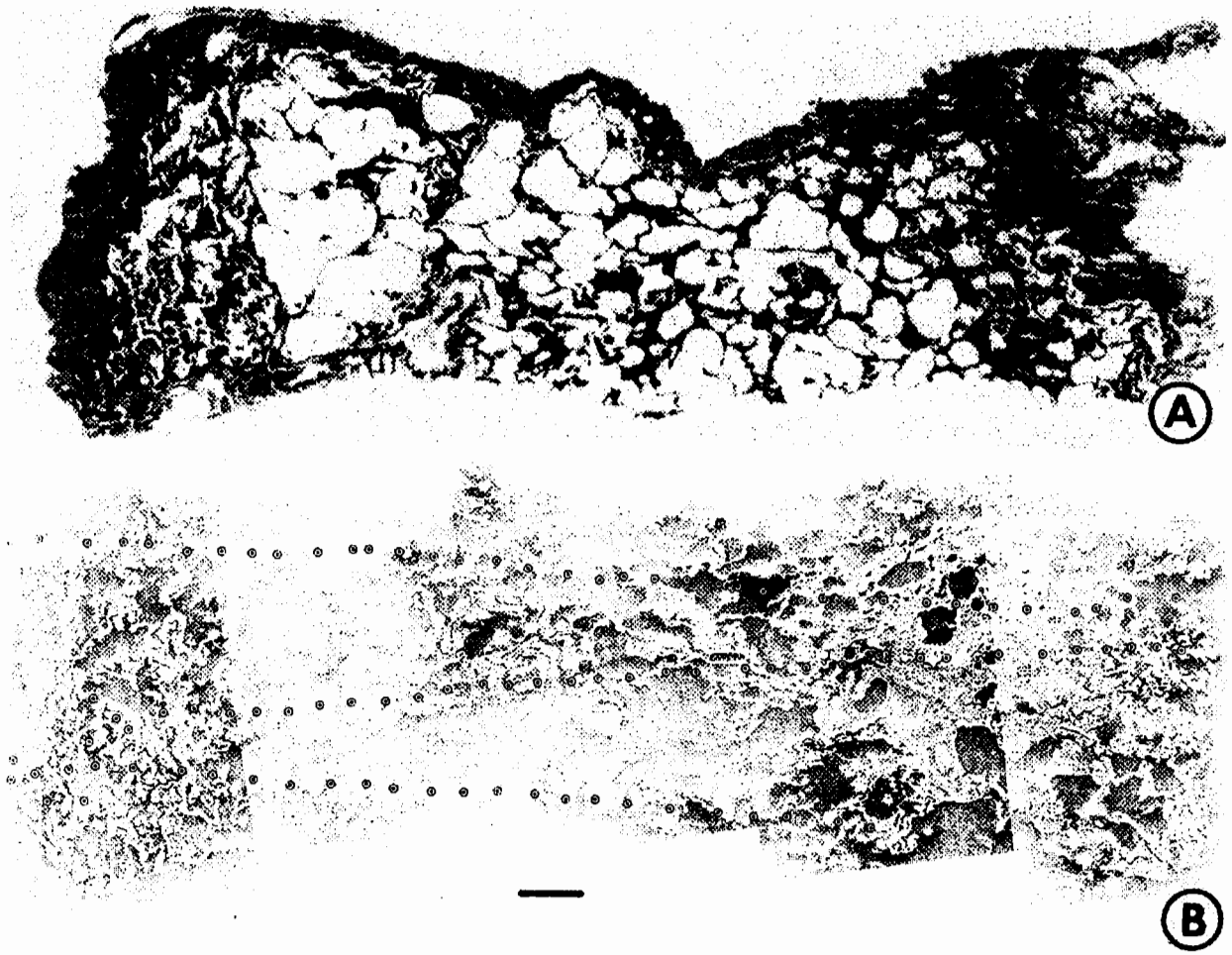

Fig. 1. Case 18. Light microscopy (A) and SEM montage (B) section on carbon. Note in B the locations (o) of the sequence of fields examined at higher magnification (see text). Marker = 200 µm.

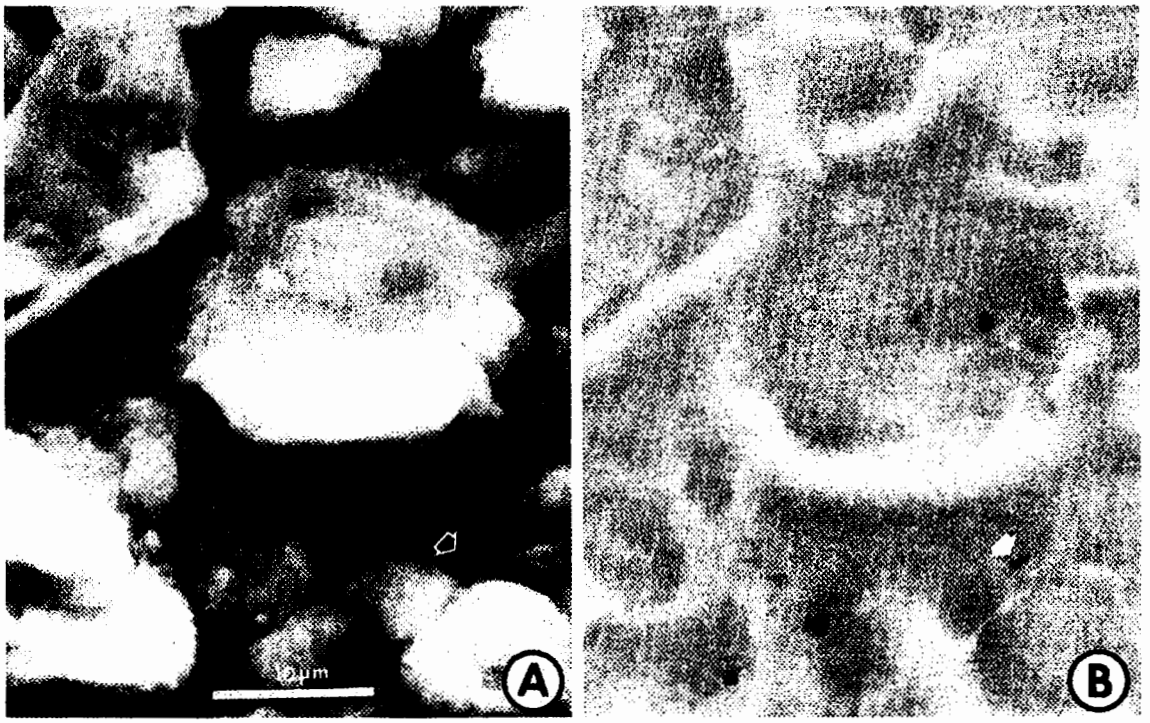

Fig. 2. Case 18. SEM, secondary electron imaging (A) and backscattered electron image (B) of one field showing several particles in the tissue (dark in this negative, reverse-contrast BSE image) and one particle (arrow) excluded from analysis as it appears on or in the carbon disc and not in the tissue.

another in case 35 (with asbestosis) than with inflation vs collapse of the nearly normal lung (case 36). Little variation is present in the concentration of exogenous particles in the six sections analyzed from case 18. All the pairs of analyses within the same case were evaluated for significance using Student's t-test. The only pairs showing significant differences were the analyses of one of the six sections in case 18 (section 1A vs 2A,B or C) ($p<0.05$), and of two different biopsy sites in case 34 ($p<0.05$).

As a further demonstration of the variability of the data, Table 2 presents the concentrations of various types of particles determined in each section of case 18.

These studies show that variability in particle concentrations is such that the same analyst or different analysts can obtain similar results, that sections can be decalcified if necessary, and that inflation of the lung is not a major factor in at least some cases (in which the majority of particles are in more rigid interstitial zones -- lymphatics near vessels or airways). Variations up to 3 fold within the same lung are reasonable and the quantitative composition of the particles seems fairly constant when determined with the volume of tissue and numbers of particles searched and analyzed, respectively.

Validation of the Method

Tissues independently analyzed by bulk methods have been analyzed using in situ quantitative microanalysis of sections to test the correlations between the two methods. Two such comparisons have been reported, one involving nickel (3) and the other beryllium (2).

Nickel in the lungs of nickel refinery workers was analyzed by bulk chemistry and representative blocks of tissue embedded and sectioned routinely. Sections were analyzed as described above in the Methods section. After determination of the total number of exogenous particles per cm^3 of tissue, the number which contained Ni was determined and multiplied by the average Ni EDXA count rate (cps) observed for those particles containing Ni. In this manner a particulate Ni cps/cm^3 was determined for each tissue section. In two cases the only remaining tissues were from 25-year old H&E stained sections (19) which had to be transferred from glass slides to carbon discs. Figure 3 shows the data from the comparison. The correlation coefficient of 0.64 is significant ($p<0.05$) given the variation in tissue sampling and other pathology

Table 1. Variability in Determination of Total Exogenous Particle Concentration.

Type of Comparison	Case No.	Mean No. Particles per Field (s.e.m.)	No. Fields
Same section, same analyst	12	2.44 (0.55)	100
		5.14 (1.36)	100
		4.34 (1.51)	100
		4.92 (1.09)	100
	13	2.95 (1.35)	100
		1.13 (0.18)	100
		1.62 (0.49)	100
Same section, different analysts	34	5.67 (1.89)	60
		4.22 (1.05)	60
Same case, different block	34	1.64 (0.36)	100
Same section, before & after decalcification, same analyst	8	1.07 (0.20)	104
		1.84 (0.39)	50
Same section, different analyst, before & after decalcification	10	1.34 (0.21)	100
		1.22 (0.19)	100
Same case, inflated & uninflated lung	35	0.75 (0.03)	200
		0.08 (0.04)	200
Same case, serial sections (1 & 2), 3 blocks (A,B & C)	18 1A	0.76 (0.18)	144
	1B	0.09 (0.15)	109
	1C	1.02 (0.23)	100
	2A	1.24 (0.28)	100
	2B	1.32 (0.56)	102
	2C	1.17 (0.47)	100

(e.g. severe emphysema in one case, which would make the determination of particles per volume or weight less reliable). The correlation coefficient is 0.80 if the case with severe emphysema is excluded.

Beryllium was studied using the IMMA since it is not detectable with EDXA. The three cases reported (2) all showed Be disease and had a wide range of chemically determined Be concentrations. The analyses with the IMMA were done similarly to those done by SEM and EDXA. The Be secondary ion count rate was recorded during etching through several areas of tissue. The maximum count rate observed was averaged for each case and plotted against the results of bulk chemical analysis of the lung tissue from the same case (Fig. 4). Note that the regression line plot shown in Fig. 4 does not intersect the point for the pure standard even though it fits three tissue "standards" well (r=0.999). Be was not detected in normal appearing tissue regions. Thus the local concentration of Be in those regions (lesions) where it was detected was higher than that observed by bulk chemistry which averaged normal and abnormal areas. With the observed background Be count rate of approximately 0.4 cps and a count rate on pure Be of 4×10^7 cps, one can determine that the actual minimum detectable Be concentration would be approximately 1 cps/4×10^7 cps or 0.1 ppm. Therefore, in the case with a bulk concentration of 0.003 ppm the actual concentration in the lesions is probably on the order of 0.1 ppm. For this type of quantitative analysis a standard reference curve is essential.

Other results of our in situ quantitative microanalysis can be compared with the literature. For example, Nagelschmidt (25) determined the amount of silica in the lungs of patients with sil-

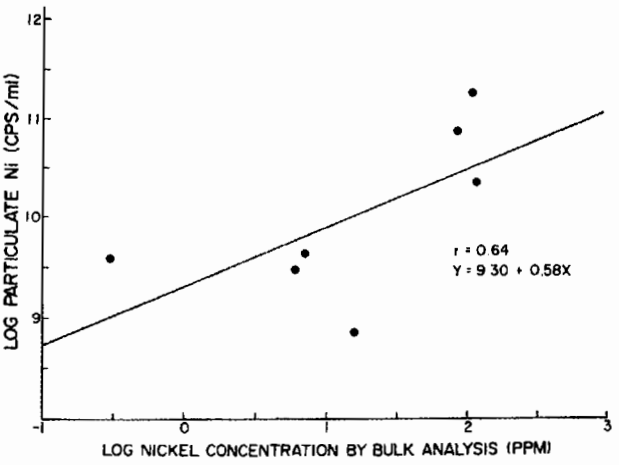

Fig. 3. Linear regression plot of calculated particulate Ni as a function of bulk chemical analysis of lung tissue (see text, and ref. 3). Log Scale.

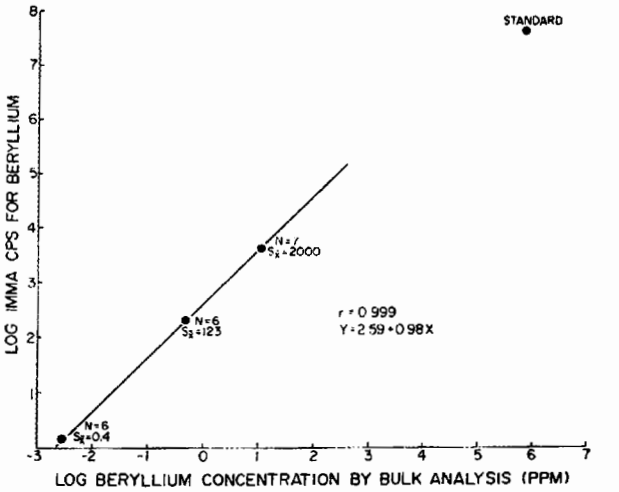

Fig. 4. Linear regression plot of IMMA results for Be as a function of bulk analysis of Be in lung tissue (see text, and ref. 2). Log Scale.

icosis to be approximately 2 grams total silica in both lungs. Our findings of 10^9 to 10^{10} silica particles/cm^3 in some cases of silicosis can be compared by assuming that the mass per particle is 2×10^{-12} gram and the volume of lungs 5000 cm^3 (5 L). Thus 10^9 silica particles/cm^3 would correspond to approximately $(10^9)(2 \times 10^{-12})$ (5×10^3) = 10 grams. These calculations, making use of several assumptions, at least show that the results are within the order of magnitude expected. Another way to look at the data is to compare quantitative microanalytical findings with epidemiology, radiology, physiology and pathology. There are scant data along these lines. Our current study of lung biopsy tissue sections (no tissues available for bulk analysis) in asbestos workers shows that the findings of numbers of asbestos fibers per cm^3 of tissue are in the same range as those reported by bulk tissue digestion studies (5). The strong correlations of tissue asbestos concentration with monitored exposures and with radiologic, physiologic and pathologic data also support the validity of quantitative in situ tissue microanalysis.

Table 2. Variability in Composition of particles* with Multiple Analyses

Sect.	# part.	Si only	Silicates	W containing	Fe containing
1A	112	0.02	0.04	0.30	0.07
1B	100	0.03	0.0	0.66	0.16
1C	99	0.04	0.01	0.77	0.17
2A	133	0.08	0.14	0.80	0.14
2B	98	0.03	0.08	0.71	0.07
2C	99	0.02	0.0	0.56	0.0

*Concentrations in numbers of particles/field (multiply by 10^8 to give number/cm^3). Same sections as in Table 1 and 3, case 18.

Results and Comments on a Series of 40 Cases

Figure 5 shows the results for 40 cases. The individual concentrations (by case number) for each major category of particle and the overall means and standard errors of the mean (s.e.m.) are indicated by a diamond. Note that the vertical scale is logarithmic, and that the s.e.m. is given as a function of the numbers of particles per standard 10,000 μm^3 field (i.e., number $\times 10^{-8}/cm^3$). The data for each individual case is listed in Table 3.

Table 4 presents some of the summary data from the 40 cases, including the grand means (of the means for individual cases) for concentrations and percentages of particles in several major categories. The overall means and s.e.m. in Fig. 5 and Table 4 include data from all 40 cases. This includes some cases with obvious disease. The overall means and s.e.m. would change if certain cases were excluded. For example, the overall mean fiber concentration of 0.09 ± 0.025 drops to 0.05 ± 0.014 if cases with known industrial exposure to asbestos are excluded (cases 19,27-35). The overall mean silica concentration is 0.36 ± 0.21, which drops to 0.067 ± 0.012 if cases with obvious silicosis

Table 3.

Case #	Pathology	Total Exogenous Particles/field (Mean (s.e.m.))
1	Pneumonia	0.23 (0.06)
2	Pneumonia	0.06 (0.17)
3	Pneumonia	0.24 (0.52)
4	Pneumonia	1.16 (0.58)
5	Pneumonia	1.12 (0.29)
6	Pneumonia	4.12 (0.64)
7	Pneumonia	2.27 (0.50)
8	Pneumonia	1.07 (0.20)
9	Pneumonia	0.51 (0.49)
10	Pneumonia	1.34 (0.21)
11	Hard metal disease	0.72 (0.18)
12	Hard metal disease	2.05 (0.74)
13	Desquamative interstitial Pneumonia	1.25 (0.56)
14	Volcanic Ash Exposure	3.91 (2.59)
15	Silicosis (Horse) (28)	2.19 (0.74)
16	Interstitial Fibrosis	1.16 (0.14)
17	Interstitial Fibrosis	0.18 (0.04)
18	Hard Metal Disease	1.07 (0.15)
19	Interstitial Fibrosis	3.61 (0.87)
20	Chrysotile Miner	0.51 (0.15)
21	Interstitial Fibrosis	1.12 (0.32)
22	Silicosis (21)	21.07 (4.66)
23	Granulomatous Disease	0.27 (0.16)
24	Acute Alveolar Damage	2.77 (1.44)
25	Infant	0.51 (0.11)
26	Hypersensitivity Pneumonitis (24)	4.04 (1.72)
27	Asbestos (5)	11.73 (2.86)
28	Asbestos (5)	1.12 (0.22)
29	Asbestos (5)	0.65 (0.18)
30	Asbestos (5)	1.81 (1.03)
31	Asbestos (5)	0.33 (0.09)
32	Asbestos (5)	0.58 (0.27)
33	Asbestos (5)	0.63 (0.13)
34	Asbestos (5)	0.63 (0.20)
35	Asbestos (5)	4.22 (1.05)
36	Normal Adult	0.08 (0.04)
37	Stillborn Infant	0.14 (0.12)
38	Granulomatous Disease	0.76 (0.51)
39	Interstitial Fibrosis, Age 6 yrs	0.08 (0.04)
40	Interstitial Fibrosis, Age 2 months	0.03 (0.17)

Table 4. Summary Data From 40 Cases

Type of Particle	Concentration (millions/cm) Median(Range)	% Particles Median(Range)
Endogenous	12 (0-290)	9.7(0 -82.0)
Exogenous	7 (3-2107)	94.0(15.8-100)
Silica	5 (0-838)	7.4(0 -66.7)
Silicates	36 (1-1010)	51.5(0.3-93.9)
Metals	24 (0-193)	32.3(0 -94.5)
Fibers	3 (0-72)	4.1(0 -77.8)

Totals: Over 6000 fields searched
Over 5700 particles analyzed

Fields per case:	126.6	57.9(s.d.)
Exogenous particles analyzed per case	120.1	64.0
Locations of particles analyzed (%):		
Interstitial	65.7	24.3(s.d.)
Airspace	28.6	24.3
Perivascular	5.7	14.5

are excluded (cases 6,15,22 and 27).

Table 5 lists the metal elements found, in order of decreasing frequency (number of cases in which element was found, out of the 40 cases). This table does not reflect the association of elements with one another. Some of the more commonly observed associations of elements within particles include Fe with Cr, Mn and Ni; Cu with Zn; Pb with Cr, Cl or Br; and Ba with S.

Our 40 cases cover a wide range of pathologic reactions. Most are not from recognized pneumoconioses, with the exception of case 22, silicosis (21) included for an example (22=lung and 22N=hilar lymph node, with even higher concentrations) and cases 27-35, a group with known asbestos exposure demonstrating a wide range of pathology (5), including one showing pathological evidence of silicosis, supported by our quantitative analytical findings (case 27). Cases 1-10 were chosen to illustrate the range of particles seen in a group of patients who died from a definite infectious cause (Legionnaires Disease). Many of these revealed underlying pathology and rather high concentrations of inorganic particulates in the lungs. These cases document the exposures antedating the terminal illness and may (unproven) indicate a group with more susceptibility to Legionella pneumophila or other respiratory infections. A recent report also points out the association between metal particle exposure and increased susceptibility to infectious pneumonia (20).

Cases 15,23,26 and 38 demonstrated granulomatous reactions. After an unrewarding search for infectious agents by microbiologic cultures and special stains, microanalysis of sections yielded some interesting results. The only metal-related granulomatous disease for which information on tissue concentrations of the agent are available is beryllium disease. The nature of the granulomatous process is different from most common pneumoconioses in that the etiologic agent of granulomas may be present in much lower concentrations, with the tissue response (the granuloma) reflecting an amplification (by sensitized immune effector cells) of the attempt to deal with an indestructible antigen (10). The tissue concentrations

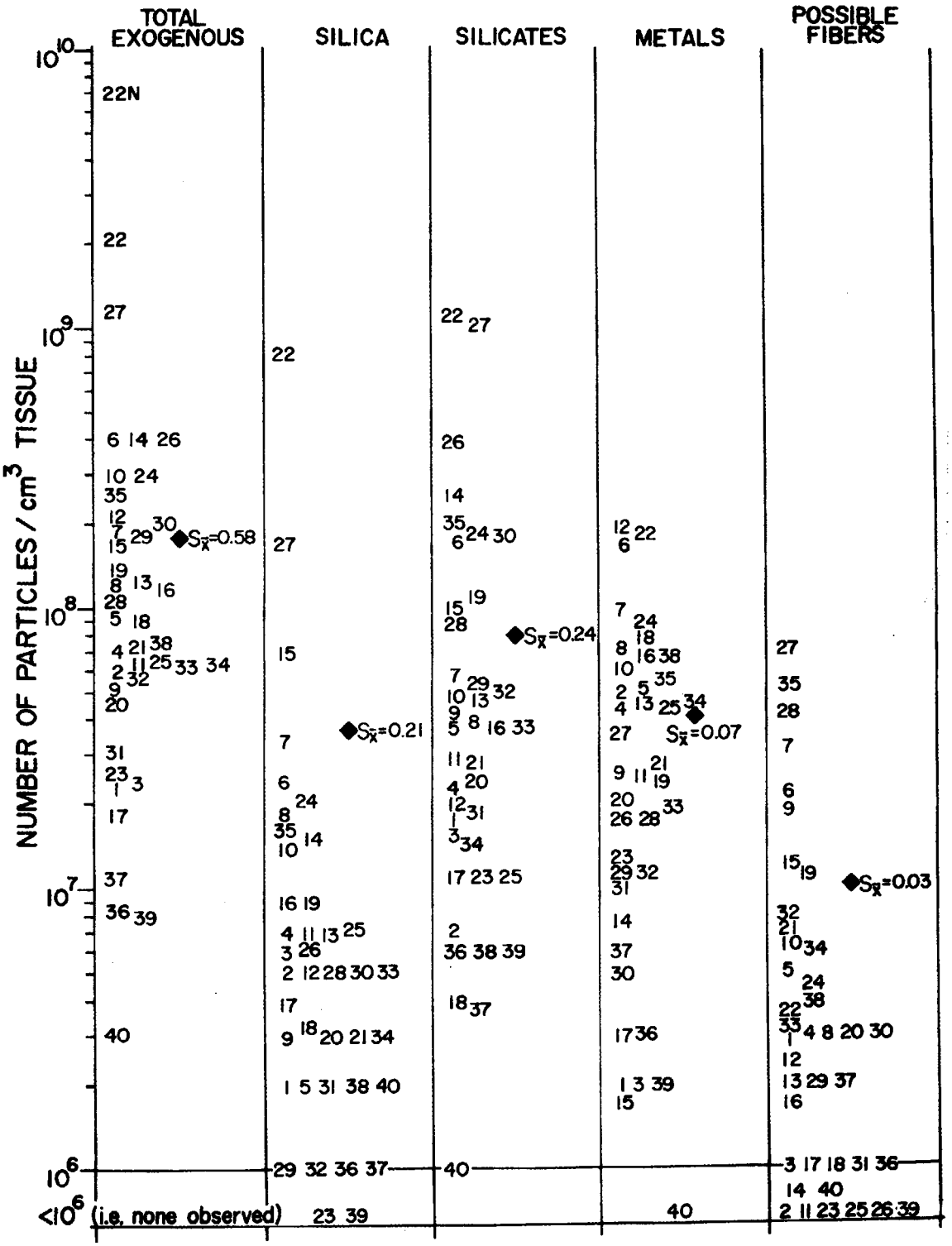

Fig. 5. Chart showing results of 40 cases. For each case the concentration (in numbers/cm^3 of tissue, log scale) of total exogenous particles, silica, silicates, metals and possible fibers (see text) is indicated by the case number. The overall mean (of the 40 cases) for each category of particles is indicated by a diamond; and $S_{\bar{x}}$ is the standard error of the mean (number of particles/field).

of the etiologic agent (discussed above) associated with Be disease (may be <0.01 ppm) are much lower than those associated with silicosis (approximately 10,000 ppm), silicatosis approximately 100,000 ppm), or even asbestosis (approximately 100 ppm).

Table 5. Frequency of Detection of Particulate Metals 40 Analyzed Cases.

Element	Frequency	Element	Frequency
Fe	39	Ba	4
Ti	30	W	3
Al	28	Ta	3
Zn	23	Bi	3
Pb	23	Zr	3
Cr	23	Ag	2
Cu	21	Sb	2
Sn	12	Hg	2
Ni	11	Os	2
Mn	5	Cd	1
Au	5	La	1
Ce	5	V	1
Br	4	Tc	1

Frequency listed is the number of cases in which the given metal was detected. This does not reflect concentrations nor associations among various metals.

In those cases of granulomatous disease in our series it is of interest that the highest concentrations of unusual metals were found, such as Al and Cr (case 23) and Al and Zr (case 38). All of these elements can be associated with hypersensitivity reactions, and Al and Zr each have been associated with granulomatous reactions (26). Further studies are required to prove causality for these materials in the individual cases. However, the uniqueness of the associations we found has suggested direction for further investigation, such as in vitro lymphocyte responsiveness to a variety of these metals, as has been done for beryllium (15).

That accumulation of ambient respirable inorganic particles in the lung begins with the first breath (or possibly in utero) is supported by our results in cases 25, 39 and 40, infants and a young child. Rare particles in macrophages have even been found in the lungs of stillborn infants. It is known that inhaled and ingested particles can be found in macrophages at sites distant from the lung and even in the urine. Thus, free particles and/or macrophages containing particles could travel from mother to fetus in utero either blood borne or in the amniotic fluid. Above average concentration of particles and particles of unusual type were seen in one infant maintained on a respirator for several days (case 25). It is possible that the respiratory therapy devices were the source of some unusual metals. A maternal history of gold therapy would also have to be excluded as a source of gold particles in fetal or newborn tissues. The cleanest appearing (pink) adult lungs examined (case 36) contained a low concentration of exogenous particles. Yet even at this low range those few particles found were not all of ambient type. A few platy talc particles suggest that there was some exposure either through cosmetic use or industrially. This documents exposure with no recognizable associated pathology at the very low tissue burden observed.

In most cases the largest fraction of the total exogenous particle concentration in the lung is the silicate group. This is not surprising since this group of minerals accounts for both the majority of particles in ambient as well as industrial exposures. Even in cases with silicosis (with rare exceptions of "pure" silica exposure) there are more silicates than silica. In cases with asbestosis the asbestos fibers (the etiologic agent) are present at lower concentrations than non-fibrous silicates. This finding serves to emphasize the importance of differentiating the types of particles in recognizing differing biological significance (e.g. fibrogenicity).

Other inorganic particles of interest in air pollution studies cannot be measured in the lungs due to solubility, (e.g., sulfates and nitrates) (16). These may be related to the insoluble inorganic pulmonary dust burden in that damage to clearance mechanisms may lead to increased retention of particles, and also as the insoluble particles may be markers of mixed exposures.

Unusual elements such as Cd and Pb in case 24, were of forensic importance. This was the only case in which Cd was found and supported the toxic exposure to fumes generated in welding on metal painted with resistant paints (containing Cd and Pb among others). This case was analyzed after exhumation and the morphology was not well enough preserved to allow correlation of pathology with analysis. The history of acute alveolar injury and lethal respiratory failure, plus the findings of recent significant exposure (Cd is relatively soluble) were enough to tie the case together. Analysis of samples of the materials on which the patient had been welding was not allowed as a result of legal maneuverings, but would be another desirable evidentiary link in such cases.

Location of Particles in Tissue Most cases showed a predominance of particles

in the interstitial or perivascular location rather than in the airspaces. The former regions are the location of the lymphatics and are the sites of accumulation of dust in most normal lungs as well. Cases in which the majority of dust was in the air spaces would be likely to have had either more recent or overwhelming exposure or some interference with the normal dust clearance mechanism. Of our cases, those with the largest fraction in the airspace were cases 4, 11, 16, 20 and 29. These were characterized by no specific type of pathology or particles in common. Thus the significance of this distributional variation is not yet clear.

In determining the relationship of the measured dust burden of the lung to the actual exposure, not only the exposure must be monitored, but there must also be knowledge of the clearance rate in the individual and the time from the exposure to the sampling of the tissue. This information is usually not available. If one assumes that a normal clearance has a half time varying from minutes (large airways) to months (distal lymphatic dust storage areas) (23), one can see that over 99% of the deposited dose will be cleared within a few weeks or months. It follows that a small decrease in clearance efficiency could with time greatly increase the pulmonary retained dust burden.

Monitored exposures are usually calculated based on one point or at most a few samples taken over a short period of time. Thus the actual total exposure to respirable dust for an individual is impossible to quantify. The only measurement which reflects all these factors is the lung burden itself. After all, the lung is the dust sampler of last resort. Whenever lung dust burden is considered it must be remembered that it represents retained dose which is a complex function of the many factors discussed above.

Light microscopic evaluation of the dust burden is useful if the particles of interest are visible and within the resolving power of the light microscope. For example, a comparison of the particles counted by light microscopy with those seen by SEM with BSE imaging was made in a series of pulmonary alveolar proteinosis cases (9,22). Here the range of ratios was from 1:1 up to nearly 1,000:1 (more particles seen by SEM than by light microscopy). The underestimation by light microscopy is especially true in the case of tiny metal fume particles which are not visible by light microscopy.

A similar comparison was made with case 22. Counts of birefringent particles by light microscopy revealed 536 particles in 300 fields of approximately 10,000 μm^3 each. The concentration determined was 1.79×10^8 particles per cm^3. The size of the particles was an average of 1.44 µm. The opaque particles were counted separately from the birefringent and showed a total of 7.0×10^8 per cm^3 in areas of alveolar and perivascular sampling and up to 70×10^8 per cm^3 in the very dusty subpleural regions. For the birefringent particles the subpleural region showed 14.8×10^8 per cm^3 and the remainder of the lung showed 0.56×10^8 per cm^3. For comparison the results of the SEM examination revealed 6×10^8 particles per cm^3 in the lung and 7×10^9 particles per cm^3 in the lymph node. The major types of particles in this case were silica and silicates which could be seen both by light microscopy and SEM.

Discussion

There is scant quantitative data available on non-fibrous particulates. Almost all work on quantitation of lung dust particulate burden has been either gravimetric (e.g. ref. 25) or limited to fibers (see ref. 32).

We have shown in the 40 cases presented above that the pulmonary inorganic fiber concentration is almost always less than the concentration of non-fibrous particles. This is so even in cases of asbestosis. Thus the importance in analyzing all the types of particulates in the lung is to allow quantitative interpretation of the relative roles and interactions of inorganic particles as etiologic agents of lung disease.

The results of two earlier studies from this laboratory utilizing in situ quantitative analysis of particulates in human lung tissue sections cannot be combined with the results of the series of cases presented here, as the methodologic details had not been refined. In the first study (9) the analyses were done at a lower magnification (4000X final), areas were not randomly searched, and no montage was made for reference to the regions analyzed. Analysis of airspaces and perivascular regions were made and showed higher concentrations of particles in the airspaces in pulmonary alveolar proteinosis cases than in the airspaces of control cases with other alveolar filling disorders. In the second study (8) the method was nearly as reported here, but the montage reference was not utilized and the system of searching, the numbers of fields searched, numbers of particles analyzed and data recording were not as exhaustive as in the final method.

Stettler and Groth (31) presented data on non-fibrous particles in three cases. These data are based on particles

recovered from tissue, and no range of concentrations in comparison cases are given. Larger numbers of particles can be analyzed more rapidly using their automated system than with in situ analysis. However, correlation with pathology is lost and the additional problem of aggregation of isolated particles remains to be solved. Losses of particles dependent on size and/or composition during the tissue ashing and particle recovery preparatory steps need to be studied further. For example, Pb-containing particles were less commonly found by Stettler and Groth than in our study.

Smoking histories were not available for all of our 40 cases. Smoking has been shown to impair clearance of particles from the lungs (14), but marked pulmonary accumulation of ambient or occupational dust can occur in non-smokers as well as smokers (30). The SEM/EDXA analyses of the silicate pneumoconiosis cases reported by Sherwin et al (30) were done before our quantitative method was developed. The exogenous dust concentration in those cases was 10^8 - 10^{10} particles/cm^3, with approximately 5% silica, for a total silica estimated at 10^6 - 10^8 particles/cm^3. The higher end of the silica estimate is in the range seen in other cases with silicosis, but no silicotic nodules were seen by Sherwin et al (30). Similar considerations apply to those cases of silicate pneumoconiosis seen in animals (12). None of the zoo animals had direct cigarette exposure. Most of the silicate particles seen in smokers' macrophages (13) are likely of ambient (soil aerosol) origin.

It would seem that the concentrations of inorganic particles remaining in the lungs of persons exposed to ambient levels of particles is orders of magnitude below those levels of pulmonary dust burden associated with significant fibrosis. Exceptions would be unusually high concentrations of ambient dust such as in humans or animals with environmental silicate pneumoconiosis (12,30) or with exposure to ambient dust containing a high concentration of fibrogenic material (e.g., silica) (28).

It is our intent that the reported quantitative data on inorganic particles in the lung will help in establishing normal ranges as well as references for levels of various types of particles found in association with pulmonary disease.

The distinction between association and causality must be remembered when utilizing such data. Criteria for causality include: 1) strength of the association, 2) consistency of the association, 3) temporally correct association, 4) specificity of the association, and 5) coherence of the association with existing knowledge (17). Results of quantitative analysis of particles in lungs are useful in evaluating all of these criteria, but are insufficient alone to prove causality. Finding of a certain type of particle which has been or can subsequently be shown to produce the same pathologic reaction in a dose-dependent manner in either humans or animals is helpful corroboration for causality. Recognition of new hazardous exposures can result in prevention of disease by reduced or eliminated future exposures.

Acknowledgements

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Discussion with Reviewers

J.A. Small: I find it difficult to believe that there are no charging problems associated with the electron microscopy of uncoated 5μm thick tissue sections at 20kV. It is possible that charging may not be a problem at very low magnification or in the BSE image. However, at higher magnifications or spot mode used for analysis charging may seriously affect the beam distribution and hence the quantitative analysis.

Authors: In contrast to standard sectioning for electron microscopy (TEM) we are using 5 μm paraffin sections from

which the paraffin is removed. A 5 μm thick plastic section would present charging problems. We and others (Scanning Electron Microsc. 1976,I:691-698, and text references 1-3,8,12,21,28 and 30) have previously described the observations with SEM of uncoated deparaffinized sections. It seems that the 20kV beam penetrates the 5 μm section to the conducting carbon substrate. Most of the tissue mass is water, which has been removed, leaving a very low density tissue. The observations that thicker sections (i.e. 10 μm or more) require conductive coating, and that charging becomes apparent if one attempts to examine the 5 μm uncoated sections with lower kV beams support this explanation. We do not use the spot mode for analysis, but a high magnification raster, with constant observation of the secondary electron image to detect any beam shifts or charging. The only particles which seem to give a problem with charging are those several μm in diameter and thickness. Most of these are not in the respirable size range. Large areas of endogenous calcification are the most commonly encountered particles which show charging in our experience.

J.A. Small: Quantitative analysis of particles in tissue is similar to analysis of inclusions in materials systems. Unless you are positive that the entire beam is contained in the particle, quantitative analysis is difficult if not impossible. Would you comment please.

Authors: The paper does not claim quantitative analysis of individual particles. As described in the text, we are quantitatively analyzing the particulate burden of the tissue, but the individual particles are analyzed in a semi-quantitative way for purposes of classification. The variations in size and matrix make absolute quantitative analysis of the diverse types of particles encountered in in situ an impractical, if not impossible, task at present. The beam is certainly not entirely contained in any of the particles we are analyzing. It passes through and is absorbed by the carbon substrate (also see next question).

J.A. Small: Why do you use direct subtraction of the carbon background since you have no idea of the percentage of the background which results from this source? Wouldn't you be better off doing some sort of local fit for each peak region?

Authors: This is partially explained in the preceding two questions and anwsers. We examine the overlayed "fit" of the Bremsstrahlung carbon background with each particle spectrum and examine the net count rates remaining for all our spectral windows. If the background fits well, the net count rates remaining in regions without characteristic peaks should be close to zero. If we observe remaining counts of more than two counts per second we first check to see if the beam current has increased by collecting a new carbon background for comparison with the one being used for subtraction. Since most of the particles we are analyzing are very small, the amount of background they generate is insignificant compared to the "constant" background from the massive carbon substrate (which is absorbing most of the beam). A small variation in absolute count rates might be important if we were attempting quantitative analysis of individual particles, but we are not.

V.L.Roggli: Many fibers within tissue sections in individuals with exposure to asbestos are much longer than the section is thick. As a result, long fibers oriented at various angles with respect to the block face may appear in two or more consecutive sections. Did you take the orientation factor into account in calulating the number of "possible fibers"/cm^3 of tissue?

Authors: As mentioned in the text, analysis of fibers in situ in 5μm sections is a recognized limitation of this method. We have not attempted to use any calculations either to determine "corrected" length distributions or fiber concentrations. The term "possible fiber" is used rather than "fiber" to allow for the possibility that some of the particles we see as fibers are plates seen on edge and that some of the particles we see are spherical, having x-ray spectra identical to those of definite fibers, may be fibers coated and/or perpendicular to the plane of the section.

V.L.Roggli: In your section on "Validation of Method," cases are described in which the in situ method is compared with the results of bulk chemical analysis. However, the methods used do not allow comparision of absolute levels, and the results of the Ni analysis show that the results by the two methods are proportionately the same (Fig. 3), but do not indicate whether 90+ % or much fewer of the Ni particles are identified by the in situ method. The same can be said for the beryllium analyses (Fig. 4). Can you comment on this problem?

Authors: The correlations shown in Figures 3 and 4 are meant to be taken as "working curves" for interpretation of

the results of similar analyses of similar tissues. For example if the exposure to Ni were not in the form of discrete particulates but rather diffuse binding of trace amounts of Ni to the tissue, one would expect to find no Ni by analysis of particulates. SEM and EDXA should not be thought of as a method for trace analysis, except when the "trace" components are present as discrete particles of high local concentration. One can make some calculations based on the determinations of the average cps/ml for Ni, for example, and compare the observed values with results on a pure Ni standard of "infinite" thickness. Under the instrumental conditions we are using, such a standard would yield approximately 5000 to 10000 cps for Ni. Making the relevant calculations one could observe that in the cases we studied, the Ni observed as particulates can account for all the Ni observed by bulk analysis. The same is true for the Be analyses done by IMMA.

V.L.Roggli: Do you have any more details on the comparison of in situ analysis of asbestos fibers with bulk digestion studies?

Authors: The asbestosis samples we analyzed in situ unfortunately did not have bulk tissue available. The availability of only a thin paraffin block of the lung biopsies made the in situ analysis the only possible way to study these rare samples. When large volumes of tissue are available, digestion and concentration of particulates on a filter facilitates analysis of much lower concentrations (10^3-10^4 particles/cm^3 of tissue) than practical by the in situ method (10^6 particles/cm^3 is the lower limit if 100 fields are examined under our conditions). We are attempting to obtain more comparative data on cases with both digestion and in situ analyses. One should note that most published data on digestive analysis is for fibers only, and also that fibers make up only a small fraction of the lung particulate burden. For example, if TEM analysis of a tissue digestate for fibers only takes one day, analysis of all the particles might take 10 to 100 days or longer.

V.L.Roggli: Would not the use of either unfixed tissue or filtered formalin for fixation of tissues be advantageous in allowing more cases to be analyzed in a shorter time (using automated image systems). Although particles in the carbon planchet would not be eliminated a "background" count could be determined and subtracted from each sample.

Authors: We have analyzed cryosections of unfixed tissue for study of soluble components. Actually, the BSE yield from these tissues is greater than that from fixed, sectioned, deparaffinized tissues, due to the residual solutes in dried cryosections. The use of filtered fixative solutions would not greatly expedite the analyses of tissues since most of the particles found outside the tissue appear to originate in the section (translocation by the microtome) or are in the substrate. Subtraction of constant "background" count determined on a blank carbon planchet cannot be used for tissues of variable porosity, since the amount of "background" seen during analysis would vary from section to section. An automated image analysis system could be used, but would require some operator supervision.

V.L.Roggli: It is bothersome that some neonates (e.g. case 25) had as many or more particles in the lungs as some adults with occupational exposure. Could this be related to contamination even though appearing "within" the tissue?

Authors: We were also surprised at this result, but subsequent analyses of other neonatal lungs have shown additional cases with evidence of particulate exposure. The source of the exposure has been identified as a corroding metal component of the respirator. This component has been replaced by a more stable material. This demonstrates once again that "occupational" exposures may begin very early in life and be impossible to elicit by history taking.

V.L.Roggli: Please provide more details or references on the method for transferring sections from glass slides to carbon or other analytical substrates.

Authors: Several methods for this have been developed in different laboratories in addition to ours. Carbon "replicas" transferring particles "extracted" from ashed tissue sections have been transferred to grids for TEM. We have used acrylic plastic to transfer intact sections or portions of sections (see text and Israel J. Med. Sci. 15:716,1979). Pickett et al presented a similar method (J. Histotechnol. 3:155-158,1980). A rapid method using collodion films was developed at McCrone Associates (available from them at 6150 So. Michigan Ave., Chicago, Ill. 60616). The quality of the transferred section is only as good as the original section and slide. If the original tissue or slide is contaminated by minute particles of dust (for example, by glass fragments common using unwashed slides) the subsequent microanalysis will be rendered much more complicated.