Morphology and Elemental Composition of Inhaled Particles in Human Lungs as Defined by Analytical Scanning Electron Microscopy (SEM) and SEM Energy Dispersive X-ray Microanalysis (SEM/EDX).

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Morphology and Elemental Composition of Inhaled Particles in Human Lungs as Defined by Analytical Scanning Electron Microscopy (SEM) and SEM Energy Dispersive X-ray Microanalysis (SEM/EDX).

By

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Abstract

Worldwide, more men and women die of lung cancer than any other type of cancer. Approximately 85% of all lung cancers are attributed to smoking. The cause of the remaining 15% of lung cancer deaths is unknown. However, during the last year, the National Toxicology Program has designated respirable silicon dioxide particles as a known human carcinogen.

Airborne particles are ubiquitous and are heterogeneous with respect to origin, size, morphology, and chemical composition. Many types of particles are inhaled and penetrate deep into the lung where they are often phagocytized by alveolar macrophages. The question arises as to whether airborne particles that are inhaled are causative agents of pulmonary diseases, including cancer.

The goal of my research was to determine whether the chemical element composition of inhaled particles in the human lung could be elucidated. This investigation was undertaken to test the hypothesis that the chemical element composition of an individual particle within a given human alveolar macrophage could be defined with an analytical scanning electron microscope (SEM) that had been configured for energy-dispersive X-ray microanalysis (SEM/EDX).

Methods have been developed in order to obtain a pure macrophage population for these studies. Fresh, surgically excised lung tissue was obtained from cancer patients who were undergoing a pneumonectomy to remove a malignant lung tumor. Different samples of the lung specimens processed for analysis by SEM/EDX included:

(a) lung tissue that had been digested chemically and the residue collected by filtration onto micropore membranes;

(b) lung tissue that had been embedded in wax blocks, sectioned, and mounted onto glass microscope slides, and subsequently de-waxed; and
(c) glass and polystyrene slides of macrophages present in touch imprints prepared using fresh lung tissues. Single particles were then probed with a Hitachi S-4000 SEM and a Princeton Gamma Tech (PGT) X-ray microanalyzer. Individual particles were viewed simultaneously in the specimen using both the secondary electron image (SEI) and backscattered electron image (BEI) modes. The X-ray spectrum that identifies a particular element is given by the PGT microanalyzer.

SEM/EDX analysis of individual inhaled particles present in the residue of chemically digested human lung specimens revealed the presence of sodium (Na), aluminum (Al), silicon (Si), phosphate (P), sulfur (S), iron (Fe) and zinc (Zn). Elemental composition of inhaled particles in thin (~ 5 μm) sections of human lung tissue was also elucidated by SEM/EDX. In subsequent studies, the same technology successfully displayed the chemical element composition of a selected inhaled particle within a single macrophage. Particularly noteworthy is that Fe was found in all specimens examined.

The results of these studies have advanced our knowledge of the chemical composition of inhaled particles and will provide the basis for future studies defining the role of macrophages and macrophage-derived factors in the etiology of different malignant and non-neoplastic lung diseases.
Dedication

To my parents who have always loved me unconditionally and with this love have allowed me to thrive and succeed. Thank you for your support and encouragement without you two and the kids I would have never gotten where I am today.

Also I dedicate this to my friends here at Carroll, you know who you are, and my friends at home, especially Mandi and Linds, you guys are always a source of guidance, love and support and I love you and thank you.
Abbreviations

SEM ........ Scanning Electron Microscopy
EDX ......... Energy Dispersive X-ray Microanalysis
PAH ........ Polyaromatic Hydrocarbons
BEI .......... Backscatter Electron Imaging
SEI .......... Secondary Electron Imaging
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Introduction and Literature Review

Airborne particles are ubiquitous. Large numbers of microscopic particles exist in localized occupational and domestic environments of both pre-industrialized cultures and industrial nations. In many environments, the particles are heterogeneous with regard to their origin, size, shape, elemental composition and many other physical and chemical attributes. On the average, an adult breathes in about 13,000 liters of air each day, and contained within this air are numerous particles. Children breathe in 50% more air per pound of body weight than do adults and may breathe in a proportionately higher number of particles. Unlike the choices we have with food and water, we have little or no selectivity in the air that we breathe. It is well documented in different epidemiological studies that certain inhaled particles are causative agents for various life-threatening pulmonary illnesses that include both non-malignant and malignant diseases.

Determining the inherent risk of air pollution has been a priority of the Environmental Protection Agency in the last couple of decades. Billions of dollars have been set aside in accordance with the implementation of the Clean Air Act. One specific subtype of air pollution is garnering increased interest in the scientific community. Airborne particulate matter has become a multi-billion dollar question and is a great public policy issue with big-stake consequences, according to Roger McClellan, a member of the EPA’s Science Advisory Board [1].

Recent epidemiological studies have shown a statistical association between airborne particulate matter (PM) and increased mortality [2]. The implications of this association, if it holds up to scrutiny, would likely cause a dramatic shift in the current
attitude towards the air that we breathe. Lippman and Bohning stated that “the average male inhales 15 kg of air each day, while he consumes only 1.5 kg of food and 2.0 kg of water” [3]. Governing organizations regulate the safety of food, beverage, medication, and practically everything else the public comes in contact with on a daily basis. Up to this point, there has been little concern and less control over the potential hazardous material that enters the public’s lungs.

Particulate matter is best defined as only those particles with a mean aerodynamic diameter smaller than 10 micrometers. This definition is suitable for respirability, as this is the limit of size that is able to penetrate to the gas exchange regions of the lungs. It has been suggested that inhalation of these particles is responsible for a number of adverse health effects due to their ability to reach the thoracic or lower regions of the respiratory tract. This penetration could conceivably induce lung inflammation and promote episodes of cardiovascular and pulmonary illness [4]. To be more precise, major implications for human health include effects on breathing and respiratory symptoms, aggravation of existing respiratory and cardiovascular disease, alterations in the body’s defense systems against foreign materials, damage to lung tissue, carcinogenesis, and premature death [5].

The spectra of airborne particulate matter includes but is not limited to dust, dirt, soot, smoke, and liquid droplets directly emitted into the air by sources such as factories, power plants, cars, construction activity, fires, and natural windblown dust. [6] The largest single source of airborne particles arising from man-made activities in urban areas is road traffic, and largest within this category is diesel exhaust particulates (DEP) [7]. Carbonaceous material is another principle component and may exist as either organic
carbon or particulate elemental carbon (PEC) also known as carbon black [8]. Of primary interest for carcinogenesis are inorganic particulates such as iron, titanium, nickel, and other heavy metals.

Various methods to determine particle content within lung tissue have been developed, however, most have only established an association between the inhalation of specific types of particles and a diagnosed lung disease. The actual harm that the particles inflict on the host has not been determined, the precise anatomical site of particle deposition is often lost in the studies, and the exact mechanisms of pathogenesis mostly remain a mystery.

Studies of human lung tissue containing inhaled particles have been plagued by technical difficulties. It may be possible, however, to develop procedures whereby inhaled particles in human lung tissue can be isolated for characterization of particle size, morphology, and elemental composition. Quantitative analysis of particle type and burden will enhance our understanding of the health risks and hazards associated with the inhalation of different types of particles. So, with this information it will be possible to implement public policies based on empirical observations rather than attempting to extrapolate data from various animal and mathematical models, which have not been validated as suitable human surrogates.

Several diseases are now known to be caused from the inhaled particles. A good example is the case study of an aluminum welder who experienced pulmonary granulomatosis [9]. This disease has also been seen in farm workers who inhaled soil-derived aluminum silicate [10] and in a painter who worked with aluminum spray paint.
Finally, a case study was done on an individual who was an aluminum-silicate packer, and pulmonary granulomatosis was found in this patient as well [12].

People who are smokers are more prone to the above disease states because found within tobacco smoke are well-recognized carcinogens (e.g., PAH, nitrosamines), cell irritants and toxins (e.g., ammonia, formaldehyde, oxides of nitrogen), carbon monoxide, and nicotine. Each of these substances contributes to the decline in efficacy of the mucociliary clearance mechanisms in the lung and finally to the destruction of the lung parenchyma itself. These components of cigarette smoke certainly put forth long-term effects on the smoker as initiators and promoters of cancer [13]. Therefore, it can be postulated that smokers, due to their impaired lung clearance mechanisms, will have significantly more inhaled particulate matter than subjects who are not smokers.

It is important to note that environmental and/or occupational hazards also exist and that these hazards play a crucial role in the inhalation and subsequent accumulation of particulate matter in the human lung. The association between occupational exposure and the eventual development of malignant mesotheliomas is nothing new and was first reported by the work of Wagner et al., in 1960 [14]. Epidemiological studies have been conducted on malignant mesothelioma in North America, and close to one half of the men and only 5% of the women had an identifiable occupational exposure to asbestos [15]. So work has been done to change the materials used in insulation in hopes that we will lessen the occupational exposure of workers. Instead we now try to use such materials as fiberglass. However, a case study done on a 56-year-old man who had a history of glass fiber inhalation for 41 years without safety precautions, such as a
protective mask, showed fiberglass fibers in his lungs. It was then suggested that these fibers had possibly contributed to his pulmonary fibrosis that had developed [16].

Based on these observations, a large number of particles would be expected to be present in the lungs of people who work in an environment, which exposes them to increased amounts of particulate matter, is to be expected. Additionally, it is thought that particles will be present in a vast majority of all human lungs to varying degrees, due to a study done in Italy on 30 patients who were not occupationally exposed to various mineral and dust particles [17]. This study found that all patients contained some degree of silica, silicates, oxides, sulfates, and metal alloys in a bronchoalveolar lavage [17].

These particles will elicit a foreign body reaction, which may progress to an acute and subsequently chronic inflammatory response. Some of these particles, particularly when present in large numbers, (i.e., bioburden) may prove to be etiologic agents and /or contributors to different pulmonary disease states, including lung cancer. Inhaled particles, granules, specs, and flakes of diverse type and origin can be observed easily in fresh, non-fixed, non-stained, and non-sectioned human lung tissue with the use of a polarizing light microscope.

Most particles will resist biodegradation, and some may be sequestered in the lung for a long period of time, possibly forever if they are asymmetrical and do not dissolve under intraphagosomal conditions [18]. Mucociliary clearance mechanisms may eliminate some particles, but are unlikely to eradicate all, particularly in people who have impaired clearance mechanisms due to habitual smoking or pulmonary disease.

The lung has many different defense mechanisms that attempt to prevent the deposition and accumulation of inhaled particulate matter in the lungs. Some
investigators have claimed that if a particle is deposited in the upper respiratory tract, coughing or sneezing will effectively eliminate it. If it is deposited more distally, in the tracheobronchial region, it will be removed by the mucociliary clearance mechanism, and if deposited in the alveolar regions, it is thought to be removed by a macrophage-mediated exoneration mechanism. However, some of these particles will resist breakdown, or biodegradation, and remain in the lungs for an extended period of time. The number of inhaled particles and the accumulation of these particles within the lung will be dependent upon the dichotomous branching of the individual’s airways and the effectiveness of the lung’s mechanisms.

It is possible that any one of these mechanisms can become impaired and compromise the lungs’ defense mechanisms, drastically decreasing the ability of the lungs to clear particulate matter. People who are habitual cigarette smokers, or have emphysema, asthma, or chronic bronchitis, are particularly prone to impaired lung defense mechanisms because these individuals experience chronic or recurrent obstruction to airflow. For people with emphysema, their elastic lung tissue degenerates, therefore impairing the strength of their cough reflex as well as their macrophage mediated clearance mechanism.

Asthma is a manifestation of localized anaphylaxis that is triggered by mast cell degranulation and mediator release in the lower respiratory tract impairing an individual’s ability to effectively clear the lungs of inhaled debris. People with asthma have hyper-reactive airways; this is an increased responsiveness of the tracheobronchial tree to various stimuli. The result is constriction of the bronchioles and an obstruction of the airway that causes difficulty in breathing manifested symptomatically by wheezing.
People with chronic bronchitis have a productive cough from chronic irritation by an inhaled substance. Chronic irritation causes submucosal gland hypertrophy and goblet cell metaplasia. This irritation stimulates a hypersecretion of mucous that entraps more debris; however, due to the impaired clearance mechanisms and eventual obstruction this hypersecretion of mucus causes, this debris is unable to be expelled from the lung.

Dr. John Pauly and his Immunology lab have pioneered critical studies in the role of fibrous and particulate matter in lung disease. In a study they found that cigarette filters release fibers which may cause an additional health risk to the smoker [19]. This study was of 12 different brands of cigarettes and the fibers that they released [19]. Using mice in this experiment and they showed how the fibers were able to withstand degradation. In addition the fibers retained the tobacco-brown color and the bright fluorescence of the tobacco tar that had been absorbed from cigarette smoke [19]. With these results the question of whether fibers released from cigarettes further jeopardize the health of smokers was raised, as well as whether or not there is a need to test components of cigarette filters for toxicity and tumorigenicity [19].

Another study examined the glass fibers that were found to contaminate cigarette filters from Eclipse. Eclipse, which is a brand of cigarette with a carbon rod, is designed to heat and not burn tobacco so as to reduce the biological activity of mainstream and sidestream smoke and to achieve a significant reduction of environmental tobacco smoke [20]. However, the carbon rod is insulated with two wrapping mats of glass fibers. In their analysis of the glass fibers they were:

(a) observed protruding from the tip;

(b) identified on the cigarette wrapping paper;
(c) viewed on the surface of the cork-appearing tipping paper;
(d) found in the pack residue;
(e) discovered lying freely on the cut surface of the fiber by both light and electron microscopy;
(f) harvested from the filter with adhesive tape; and
(g) displaced when Eclipse was smoked mechanically [20].

Puffing on Eclipse discharged glass fibers and glass particles from the filter into the smoker's mouth, this led to these bioresistant particles and fibers being ingested or inhaled [20]. It was concluded that there is a potential and unnecessary health hazard to uninformed consumers [20].

Studies in Dr. Pauly's lab were also undertaken to determine whether inhaled plant and plastic fibers were present in human lungs and, if so, whether inhaled fibers are also present in human lung cancer [21]. They found that morphologically heterogeneous fibers were seen repetitively in freshly excised human lung tissue using polarized light [21]. These inhaled fibers were present in 83% of nonneoplastic lung specimens and in 97% of malignant lung specimens [21]. It is thought that these bioresistant cellulosic and plastic fibers are candidate agents in contributing to the risk of lung cancer [21].

Human lung tissue can be digested chemically, and the residue of the digestion collected onto micropore membranes so as to enable the critical analysis of these particles. The size and morphology of diverse types of inhaled particles can be obtained with analytical scanning electron microscopy (SEM), and the elemental analysis of each inhaled particle can be acquired by SEM Energy Dispersive X-Ray Microanalysis (SEM/EDX). Macrophages that ingest these foreign particles may be found, and by
using SEM/EDX the particles within may be chemically identified. Macrophages from fresh lung tissue can be isolated, then counted and digested. The field of particles left behind can be quantified, and relations can be made between the number of macrophages and the number of particles [22].

A pilot test using chemically digested human lung tissue samples allowed for particles to be collected on a micropore membranes. Matt Swift and I performed this test to demonstrate that lung tissues contained particulate matter. The test proved that particulate matter could be isolated and analyzed with SEM and SEM/EDX, and was the beginning of my study. Used, as a control in this study, was fetal bovine lung tissue because it demonstrated that particles were indeed localized solely to the lung tissue in question and not a product of contamination or improper SEM readings.

For my research project I studied human lung tissues and the particles that they contained, examined the particles without organic matter by way of digestion, and studied tissue either embedded in paraffin, touch imprints made of fresh tissue or macrophages isolated from fresh and fixed lung tissue. The project had the following objectives:

1. the determination of whether a procedure can be developed whereby human lung specimens can be digested and inhaled particles isolated for analysis by SEM/EDX,
2. the determination of different types of elements found in inhaled particles,
3. the isolation of alveolar macrophages (AM) from distal lung tissue and the identification of particles within,
(4) finally the digestion of these AM and organic matter in order to count
and identify the particles that they contained.
Materials and Methods

Human Subjects.

Lung tissue samples used in these experiments were obtained from human subjects, a majority of whom had been clinically diagnosed with lung cancers. Patients without diagnosed lung cancer were also included. Donors were patients at the Roswell Park Cancer Institute (RPCI), Buffalo, NY. The Clinical Investigations Committee at RPCI (CIC 97-10, CIC 92-06) approved the Human Specimens/Patient research protocol for this study. Consent was obtained from all RPCI patients through proper channels.

Fresh Human Lung Tissue (RPCI).

Lung samples obtained from the RCPI patients included in this study were residual tissue from patients undergoing surgery for removal of a lung tumor. The specific surgical procedure undertaken was one or more of the following: lobectomy, pneumonectomy, thoracotomy, wedge resection, and lung resection.

Tissue samples from the RPCI surgical patients were obtained with written informed consent under the above approved investigative protocols. Samples of the surgically excised adjacent non-neoplastic lung (herein referred to as residual) tissue were obtained during the procedure and placed under the control of RCPI’s Tissue Procurement Program.

Under the Tissue Procurement Program at this Institute, portions of the residual tissue were collected for the specific purpose of this study. Specimens were assigned a study number and placed in new, clean, wide-mouth, polypropylene specimen jars (Nalgene, Rochester, NY; No. 2118). The jars were then transported, within approximately one hour following excision, to the research laboratory. All necessary
precautions were taken to avoid contamination of the excised lung specimens with extraneous material. Contamination avoidance will be discussed in later sections.

**Laboratory Inventory of Lung Specimens.**

Upon delivery to the research laboratory, specimens were given Lung Study Numbers, which were made irrespective of previous identification. RCPI specimens were categorized with the letter “L” followed by a sequential number of delivery. After this inventory was made, the samples contained no patient identifiers to ensure confidentiality in the ensuing experiments. After study numbers were assigned, human lung study protocol sheets were completed to record information regarding the lung specimens such as: initial procurement or identification number, date, time of excision, weight, and gross morphology. All relevant material regarding patient history was kept with the protocol sheets.

In most instances, examinations reported herein were performed using fresh tissue samples. When it was not possible to process and examine the tissue samples while fresh, the specimens were refrigerated, usually for less than 48 hours.

**Protection Against Examiner Contamination.**

Before handling any equipment or tissue samples, the examiners hands were washed, rinsed, and air-dried. Powder-free latex gloves (Diamond Grip™; Microflex Medical Corp.) were used to both protect the materials and the examiner.

**Use of a Laminar Flow Hood.**

Examination and handling of all tissue was conducted under the protection of two six-foot wide laminar flow hoods (airflow rate, >100 ft/min). Both hoods were used in a given experiment (Fig. 5). Each hood had intake and exhaust HEPA filters with a
certified ≥ 0.3 μm particle exclusion (Labgard). The laminar flow hoods provided safeguards against potential airborne fiber contamination within the laboratory.

Surfaces of the hoods were cleaned with a 95% ethanol (e.g., ethyl alcohol) solution and a synthetic (e.g., cellulose) sponge used exclusively for this cleaning. The fan was allowed to run to purify the air before placing any surgical instruments, tissue samples, or other items into the protection hoods.

Flaming of Instruments to Remove Contaminants.

After preparing a sterile environment in which investigation of the lung tissue samples could proceed, proper measures were taken to ensure that potential contaminants were removed from the surgical instruments.

A conventional 10-x 20-cm stainless steel surgical instrument tray was washed thoroughly with a detergent and water. It was then flushed with deionized water and, without drying, placed in a laminar flow hood. Similarly, an 8.5 x 10 cm covered glass-staining dish (Wheaton No. 900200) was cleaned, placed within the hood to air-dry and filled halfway with 95% ethyl alcohol. All required instruments (a pair of forceps, sharp-nose scissors, glass slide, weight boat) were thoroughly washed in the same manner as above and immersed in the ethanol solution. Afterwards, the ethanol-soaked instruments were ignited within the hood via a previously lit flame. Instruments were placed within the surgical tray after flaming. This procedure burned and eliminated many extraneous contaminants (e.g. fibers and particles) that may have been present. The instruments were allowed to cool before use. The efficiency and effectiveness of this procedure for successfully destroying particulate matter, was demonstrated previously by laboratory technicians.
Collection of Lung Tissue Samples for Analysis.

The specimen jars were brought into the hood and then opened. Using cleaned, flamed forceps and a scalpel, the tissue samples were grasped and incisions made exposing the perceived interior-most portions of the specimens. From this location within the specimens, scissors were used to excise fractional sections of tissue samples for analysis. The attempts made to collect the samples from the innermost part of the specimens were done to again minimize the possibility of contamination of the tissue samples. Internal samples would likely be devoid of such artifacts. Control samples were handled in an identical manner.

The excised test and control samples were placed within cleaned and flamed aluminum foil weight boats. The samples were weighed with the use of a Mettler AC 100 analytical balance that had been positioned in the laminar flow hood and tarred to reflect the weight of the empty aluminum foil weight boats. The gross wet weight of the internal samples was then recorded. For the purpose of chemical digestions, approximately 0.5 g (specimen weight range, 0.48 to 0.51 g) of lung tissue was required.

Chemical Digestion of Tissue Samples.

After sample weights were recorded, the excised portions were placed into guaranteed sterile 50-ml conical polypropylene tubes (Falcon®). The Falcon tubes were then filled with 12-15 ml of filtered digestion fluid, a proprietary formulation that had been stored in a sterile environment. The tubes were capped, labeled and placed on a Bellco orbital shaker. This machine provided constant motion to assist and expedite the digestion fluid as it broke down the tissue samples. The Falcon tubes remained on the orbital shaker until no visible sections of tissue remained, which was anywhere from 10-
45 minutes. Upon completion, 35 ml of hospital grade sterile water (Baxter Healthcare Corp.) was added to each Falcon tube.

**Filtration Apparatus.**

A technique was developed for the use and storage of a micropore membrane filtration apparatus (Wheaton). Before each use, all vital portions of the filtration apparatus were properly cleaned and sterilized (Allart-Fagan, look up in matt’s # 25?). The filtration apparatus was then assembled within the laminar flow hood. Petri dishes, which would later hold the membranes for drying purposes, were also cleaned, sterilized, labeled, and stored within the hood.
For filtration, a fitted glass base was slipped into a hole of a rubber stopper that had been positioned securely atop a clean flask. For the purpose of this study, two different types of micropore membranes were used for particle analysis. The first, a 25 mm diameter, 8.0 μm pore size cellulose membrane (Millipore) was used for light microscope analysis to provide the investigator with rudimentary knowledge of the particle content of the digested lung sample. The second membrane, a 25 mm diameter, 0.4 μm pore size polycarbonate membrane (Millipore) was used for the primary SEM/EDX analysis. The same procedure was used for both types. The membrane was rinsed by dipping it twice in sterile water and was then placed on the glass base. At all times, the membrane was handled with sterilized tweezers. A glass chimney was then secured on top of the membrane with a section of Parafilm. Using thick-walled rubber tubing and a conventional sink aspirator, a constant and uniform vacuum between the sink and the apparatus was created for the purpose of aiding filtration.

The contents of the Falcon tubes were divided approximately in half between the two membranes. The digested lung residue was slowly poured into the chimney and filtered through either membrane. After the portioned amount of digest had been passed through a membrane, the chimney was rinsed with sterile, bottled, hospital grade water to clear remaining particles. The membrane was transferred into the proper petri dish and placed into a 56° C oven to dry for a minimum of two hours. The chimney and base were disassembled and cleaned before filtration with the next membrane and the above procedure was repeated for all test and control samples.
**Light Microscopy of the Membranes.**

The cellulose membranes were mounted onto 2- x-3-inch pre-cleaned microscope slides. All mounted membranes were viewed with a binocular microscope having vertical and transmitted light optics (Reichert-Jung). This microscope was configured to permit viewing with white, fluorescent, phase contrast, and polarizing light.

All cellulose membranes from test and control samples were observed prior to SEM/EDX analysis of the polycarbonate membrane. Written and photographic documentation of observed particle contents were obtained.

**SEM/ EDX of the Membranes.**

The polycarbonate membranes were transferred to sterile tissue culture dishes (Falcon Plastics) and brought to the South Campus Instrumentation Center at SUNY/AB. The membranes were mounted with double-sided carbon discs onto aluminum SEM stubs. The carbon tape was vital in that it provided a reliable conductive background with no characteristic x-ray signature. The stubs were then coated with approximately 20 nm of evaporated carbon (Denton DV502 evaporator) to prevent charging (Fig. 2, Fig. 4a, Fig. 4b). At all time the stubs were protected against contamination and were stored in closed vessels.

All prepared membranes from test and control samples were examined and analyzed morphologically with a Hitachi S-800 field-emission scanning electron microscope at an accelerating voltage of 25 kV. Inorganic particles were localized using the backscattered electron imaging mode (GW solid state detector), and correlating X-ray spectra were collected with a PGT x-ray microanalyzer and displayed from a connected Apple Macintosh. All spectra were printed, saved, and stored in confidential files.
Similarly, secondary and backscattered electron images were digitized and stored with additional Apple Macintosh software, printed, and saved.

**Section Analysis.**

A second study was undertaken to begin analysis of inhaled particles in conventional Hematoxylin and Eosin stained paraffin wax sections of human lung tissue. Upon receipt of fresh human lung tissue a portion of it was fixed in formalin solution and taken to Mary Vaughn in Histology, where she would cut wax block sections that contained our tissue (Fig. 3). These sections were then properly labeled, mounted on a microscope slide, and stained using Hematoxylin and Eosin, leaving the adjacent section unstained. The unstained section was then de-paraffinized and mounted on a carbon stub for use in the SEM. The conventional section was analyzed with a stereo-zoom microscope and digitized images were prepared to create an oversized montage of entire fields of interest. With the montage as a guide, the SEM was able to examine the same fields on the adjacent section mounted on the carbon stub. Particles were then analyzed by our SEM/EDX for elemental composition.

**Touch Imprints for Macrophage Analysis**

Using freshly excised human lung tissue, a portion was cut of the interior portion of the sample and under the protection of the laminar flow hood the tissue was then touched multiple times onto glass and polystyrene slides (Fig. 5). These were then allowed to air dry under the hood and viewed under light microscopy.

Touch imprints were also used in the SEM/EDX (Fig. 1), after carbon coating to locate and analyze isolated alveolar macrophages for ingested particulate matter. These techniques proved quite valuable. It allowed the laboratory to view and appreciate the
morphology of the intact macrophage and actually see the particles within thereby assuring us that the particles found were not due to contamination.
Results

Summary of Lung Samples.

In this study, residual lung tissue from patients undergoing a lobectomy or partial pneumonectomy for the removal of a lung tumor were critically examined for the presence of inhaled particulate matter.

Freshly excised non-neoplastic human lung tissue was examined with the naked eye upon receipt in the laboratory. The non-neoplastic lung tissue varied from a dark red to a pink color and had a spongy consistency. Carbon-like deposits were readily observed and were present in most lung samples.

Experiment and Surgical Controls.

A control has been developed for the process of experimental investigation (Stegmeier, 1997). The ideal control tissue was determined to be a specimen that had never been exposed to airborne contaminants and, therefore, would have no inhaled pollutants. The tissue selected, near-term (~70 lbs) fetal bovine lung, was deemed an appropriate control tissue to assess the potential for airborne fiber contaminants. This control provided the laboratory with an abundant amount of lung tissue that had not been exposed to inhaled air. This tissue was used as a control each time human lung tissue samples were handled to allow for the determination of possible contaminants.

The fresh, near-term, fetal bovine lungs were obtained with approval and certification from the U.S. Department of Agriculture (USDA). The lungs were donated (a gratis) by a licensed large-scale commercial meat packing plant. Fetal calf serum for various biological studies was also collected at this facility; thus, the technical staff was familiar with proper procedures for handling bovine fetuses for biological studies. Upon
receipt, the fresh fetal bovine lung was bright blood red, fleshy in appearance and relatively spongy in consistency, no visible carbon particles were observed. The tissue was placed in a laminar flow hood and sectioned (~ 50 g). Each piece was placed in a plastic vessel and stored frozen at -20° C. As mentioned above, prior to any experimentation on human lung tissue samples, an appropriate amount of fetal bovine lung specimen was thawed at room temperature and examined in the same manner.

Used in combination, the above control provided the research laboratory with adequate assurance of the “purity” of human lung tissue samples from the point of removal from the donors to the time experimentation was complete. With this in mind, particles observed had been clearly collected from the lung samples and not from artifacts of the experimental process. This was due to the fact that the fetal bovine lung contained no visible particles. Exact techniques of contamination prevention will be addressed below.

**Chemical Digestion of Tissue.**

The Immunology lab of Dr. John L. Pauly, has developed a simple procedure whereby exogenous particles retained in the human lung can be collected and analyzed individually by SEM/EDX. Our procedure involved the chemical digestion of fresh human lung tissue, which destroys organic matter but does not affect mineral composition significantly. This technique has proven to be suitable for processing a variety of specimens including human lung, fetal bovine lung, and various types of human non-lung tumors (e.g. leiomyosarcoma) used as surgical controls for contamination. The procedure developed for the chemical digestion of tissue proved to be inexpensive, expedient, efficient, and reproducible. The lung digestion protocol takes
advantage of the proteolytic activity of naturally occurring enzymes. In most instances, tissues from different sources could be digested within 15 to 20 minutes. Times varied with respect to the degree of freshness of the sample as well as the particle burden of the sample. Fastest digestion times occurred with non-refrigerated samples. The total time of the digestion procedure, which included sample collection, digestion, filtration, and membrane isolation, was less than two hours. Membranes were allowed to dry in a sterile environment before analysis with the SEM.

Analysis of a known asbestos fiber discovered on a membrane after complete digestion showed that this technique would not alter morphology or chemistry of the inorganic contents within the sample. Further analysis, as described below, demonstrated that this digestion process provided a membrane that was very suitable for analysis. Particles were well distributed without significant clumping or aggregation. Carbon coating of the membranes prior to SEM analysis was completed with ease and X-ray microanalysis was conducted without difficulty for all particles greater than 0.3 μm in diameter.

**Inhaled Particles in Human Lung Tissue.**

The primary purpose of this investigation was to achieve the following objectives:

(1) to determine whether a procedure can be developed whereby human lung specimens can be digested and inhaled particles isolated for analysis by SEM/EDX;

(2) to determine different types of elements found in inhaled particles;
(3) to isolate alveolar macrophages from distal lung tissue and identify particles within; and

(4) to digest these alveolar macrophages (AM) and organic matter in order to count and identify the particles that they contained.

In this study freshly excised lung samples were obtained from patients undergoing lung surgery. Both malignant and non-neoplastic samples were usually received in the lab in less than one hour and completion of the experiments were done in around two. The samples that were seen in the laboratory were of differing histological types, including adenocarcinomas, squamous cell carcinomas and adeno-squamous carcinomas. The samples that were removed from these lungs were not always taken from a particular area and in each case particles were found. It was concluded that inhalation of particulate matter is not limited to the upper airways.

Three commonly practiced, simple, and efficient techniques that allowed for the isolation and viewing of inhaled particulate matter from fresh human lung tissue. This study used two of these techniques,

(1) compressing it between two slides (dual chamber squash technique) and

(2) chemically digesting the tissue and collecting the residue via filtration onto a membrane.

In addition a new technique, touch imprinting was also implemented.

All of the aforementioned techniques were found to be suitable for critical examination of inhaled particulate matter with the use of a conventional microscope utilizing white light, fluorescent light, and polarizing light. The dual chamber squash technique was beneficial, in that it allowed for the viewing of freshly excised human lung
tissue for signs of inhaled particles. However, due to the size of the second slide, this technique reduced the working distance of the microscope. This did not permit us to reach a magnification of greater than 20X, but it was not necessary to go any further than this to see the inhaled and ingested particles in the lung and macrophages.

After the lung tissue samples were prepared they were examined with epifluorescence microscopy. Utilizing the epifluorescence microscope with a FITC or TRITC filter configuration, the examination of lung tissue samples from different patients showed a strongly fluorescent lung stroma, due to the autofluorescence of the tissue. In addition, macrophages in these lung samples were also observed to strongly fluoresce. This high level of yellow-green fluorescence in the lung stroma as well as in the macrophages present within this stroma is unique to the tissues of the lung when compared to similarly examined tissues from other organs in the human body. This fluorescence is unique because it was determined to be secondary to the inhaled particulate matter and polycyclic aromatic hydrocarbons present exclusively in the lung tissue.

The particles were quite varied in all aspects be it morphology, size or chemical makeup. They ranged in shape from chip-like pieces to spherical balls. As far as size they again varied anywhere from 1 μm to 5 μm (Fig. 6). Particles were chemically determined and they contained few to many different elements, including Fe, Al, Na, Si, P, S and Zn, being the most common in one particular study (Table 4). Each of these ranged in values but comparatively speaking Fe was the most prominent.

By using certain techniques, such as teasing lung tissue in formalin (which allows for intact macrophages) we were able to isolate and analyze alveolar macrophages for
inhaled and ingested particles. Another technique used was the focusing, or concentrating of large numbers of macrophages, onto a micropore membrane by use of a filtration apparatus similar to the one used for the digestion method. There were two ways in which we could view the macrophages, either fixed or unfixed. By using the fixed, in which are macrophages are preserved intact morphologically, due to the formalin solution they were soaked in, it was possible to see the complete integrity of the macrophage membrane. However, with this comes the problem of obstructing the internal particles from view. When leaving the membranes unfixed it was easy to see the ingested particles within the macrophage due to the collapse of the membrane around them (Fig. 6). At the same time it was difficult to get the cells’ morphology. A combination of both methods seemed to be the most beneficial.

After AM isolation we put the macrophages onto glass or polystyrene backgrounds for use in the SEM/EDX. With the advanced technology of the Princeton Gamma Tech microanalyzer the exact location of analysis within the particle was pinpointed. Readings were taken of ‘blank’ membrane, red blood cells, and the carbon stub in order to compare these with the readings obtained from the particles within the membrane (Table 4). Upon analysis of the data it was easy to define these chemical components. The particles ranged again in composition, anywhere from containing Fe to Ca to Cl. Once more, virtually every particle contained a significant amount of Fe.
Discussion and Conclusion

The findings that presented in this paper are significant in that they document the presence of various types of chemically defined particulate matter in fresh human lung specimens. Moreover, inhaled particles were observed in all of the human lung specimens and were seen in most subjects with a high frequency.

These findings suggest that individuals are exposed to a significant health risk. Inhalation of particulate matter is unavoidable and that these particles can activate host defense mechanisms to induce a foreign body reaction.

In this study (Figure 9) a significant amount of Fe was found in all ten particles that were analyzed from chemically digested human lung tissue. Fe is thought to play a vital role as a catalyst in the Fenton Chemistries and Reactive Oxygen Species (ROS) (Fig. 8). The superoxide anion is one of the most common and important oxygen free radicals. When Fe$^{2+}$ is present the superoxide anion free radical can be dismutated to form H$_2$O$_2$ and the highly reactive hydroxyl radical (*OH). Oxygen free radicals play a central role in the initiation of cellular injury that leads to the development of several lung diseases [9]. Generation of excessive amounts of ROS may lead to many problems, including: "stimulation of the inflammatory process; secretion of chemotactic factors, growth factors, proteolytic enzymes, lipoxygenases, and cyclooxygenases; inactivation of antiproteolytic enzymes; and activation of oncogenes and transcription factors[11].

One objective of my project was the further development of the SEM/EDX technology so that a standard H&E section can be analyzed with light microscopy and subsequently analyzed with SEM and SEM/EDX, to make a pathologic diagnosis. By this improvement, those specific particles with known chemical composition can be
correlated to that diagnosis and shown to play a significant role in pathogenesis. The characterization of chemically defined inhaled particles within the lung will prove valuable for future studies assessing lung cancer and other respiratory diseases associated with the inhalation of airborne particulate matter.

Another method I tested was the analysis of alveolar macrophages and the particles that they contain while intact. Touch imprints of fresh lung were invaluable because it allowed us to view intact macrophages and get an understanding of their morphology when containing ingested particulate matter. Using fixed tissue, which had been allowed to soak in a formalin solution, permitting the retention of the outer membrane integrity, could also make touch imprints. Which gave a much better view when using the SEM/EDX. I am also trying to isolate the macrophages and collect a large number of them, so that in the lab it will be possible to chemically digest away the organic material and focus solely on the particles that were within them and determine their chemical makeup.

In summary, inhaled particles were observed in all lung specimens examined. There is an increased amount of particulate matter in people who have an impairment of their alveolar macrophage mediated clearance mechanism. Evidence suggests that the volume of particles phagocytized by alveolar macrophages is the most critical factor in causing impairment in clearance function. It is in fact this high volume of inhaled particulate, which has been demonstrated to lead to a lung which is “particle-overloaded.”

Upon completion of this study four conclusions were drawn. The chemical element profiles of inhaled particles in digested human lung specimens were determined. The morphology and chemical elements in wax block sections of human lung tissue were
defined. The technology for SEI, BEI, and SEM/EDX analysis of a single inhaled particle within a given human alveolar macrophage was further developed. Finally, Fe in macrophage-ingested particles was identified.

More research is needed for a complete understanding of the basic mechanism leading to non-fibrous particle-induced tumorigenesis in the lungs of humans. Also, future experiments can be done on an entire cohort of people and not just on lung tissue acquired from patients at Roswell Park Cancer Institute.
Fig. 1. View of the Hitachi-4000 scanning electron microscope configured for X-ray microanalysis (SEM/EDX) that was used for the identification of elements in inhaled particles present in human lung tissues.
Fig. 2. Carbon-coated touch imprints of human lung tissue, are shown being inserted into the chamber of the Scanning Electron Microscope shown in Figure 1.
Fig. 3. Preparation of the wax block slide of human lung sample. After removing the wax, the lung tissue was probed with the SEM/EDX in order to determine the chemical element composition present in the inhaled particles.
Fig. 4a. Coating of the lung samples with carbon in order to prevent electrical ‘charging’ that may occur during analysis of the specimen with the Scanning Electron Microscope.
Fig. 4b. Close up view of the carbon coated samples.
**Fig. 5.** View showing the preparation of touch imprints of a fresh, surgically excised human lung specimen from a patient with lung cancer. The SEM/EDX (Fig. 1) was used to probe the alveolar macrophages of the lung (Fig. 7) to generate an X-ray spectrum of the chemical elements present in ingested particles (Fig. 8).
Fig. 6. A view with a Scanning Electron Microscope of a single human lung macrophage that has ingested a number of particles, some of which have been labeled "a" to "h". The chemical element composition of each of these particles was established by SEM/EDX. The X-ray histogram defining the chemical elements of particle "a" is shown in Fig. 7.
Fig. 7. Shown here is an X-ray histogram of a single particle within a human lung macrophage. The blue histogram is that of the glass slide (background). The red histogram is that of the phagocytized particle.
Fig. 8. Shown here is a histogram representing background readings of red blood cells.
Fig. 9. Shown here is a histogram of a polystyrene background SEM reading.
Fig. 10. Shown here is a histogram of a blank carbon stub reading. The last three figures were done in order to compare relative amounts of elements contained in actual sample readings.
Fig. 11. Iron (Fe) was identified as a major element. This Fe was in a single particle present in a touch imprint of a fresh human lung specimen.
Table 1 — A table displaying different chemical elements that were identified in inhaled particles present in a human lung specimen that had been digested chemically. Noteworthy is that all the inhaled particles contained iron (Fe).
Literature Cited


