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Relationship of Clearance and Deposition of Silica to Histological and Immunological Changes

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ABSTRACT

The alteration of B lymphocyte immunocompetence was studied in the mouse following exposure to silica dust over a period of 39 weeks. The thymus-independent anti-Escherichia coli lipopolysaccharide (LPS) response of mice after aerosol stimulation with whole E. coli 055:B5 was significantly suppressed in the spleen at all time points. Enhancement at nine weeks and subsequent depression of the immune response to LPS occurred in the mediastinal lymph nodes (MLN). This effect was not seen, however, when the silica-stressed lungs were bypassed by intravenous injection of antigen. The development of silicotic lesions in the mouse following exposure to silica dust over a period of 39 weeks was studied histologically. Using polarized light silica-laden aggregations of dust cells were observed at 24, 33, 36, and 39 weeks. Energy-dispersive x-ray analysis (EDXA) confirmed the presence of silica dust in the lungs. This technique also identified the presence of submicroscopic silica particles in the spleen. Granulomatous and fibrotic changes were seen in the lungs and MLNs at 39 weeks. Perivascular and peribronchiolar lymphocytic cuffing was widely observed, particularly in aerosol immunized lungs. A greater number of macrophages was found in the bronchioles of the silica-exposed animals. Using scanning electron microscopy, infiltration of macrophages was observed adjacent to blood vessels and respiratory airw

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INTRODUCTION

The cytotoxic effects of particulate pollutants on the cells and the mechanisms of the immune system have warranted investigation because of their potential health hazard. In particular, a high incidence of respiratory and neoplastic diseases has been reported in individuals chronically exposed to mineral dusts containing particles of silicon dioxide (SiO_2). SiO_2 is a causative factor in the pathogenesis of coal worker's pneumoconiosis and silicosis. The pneumoconioses are a group of diseases caused by the inhalation of mineral or organic dusts. Silicosis is a pneumoconiosis caused by the inhalation of the particulate SiO_2. In recent years there have been various inhalation studies with silica to demonstrate that silicotic lesions could be experimentally induced in animals. However, these inhalation studies did not attempt to investigate the mechanism of silica's action on cells and tissues.

The main purpose of this investigation was to obtain a clearer understanding of how silica affects cells and tissues. Thus, this study attempted: (1) to produce the chronic pathological condition of silicosis in an animal model; (2) to evaluate the capacity of silica-exposed mice to mount an immune response against an antigen during chronic silica exposure; (3) to evaluate the progression of the silicotic lesion by means of light microscopy and scanning electron microscopy; and, (4) to localize silica in cells and tissues by means of polariscopic light microscopy and energy dispersive x-ray analysis.

LITERATURE REVIEW

General Features

Silicosis is a chronic pulmonary disease. It is produced as a result of the inhalation of particulate matter containing sufficient silicon dioxide in chemically uncombined form to be pathogenic over a long period of time. The mining industry and other industries, such as quarrying, stone cutting, abrasive blasting, vitreous enameling and foundry work, cause airborne dusts containing various concentrations of silica dust at the working site. Although regulations are now enforced both to reduce the dust hazard and to compensate victims of silicosis, it is still a common occupational hazard.

Factors Determining the Occurrence of Silicosis

Among the factors that determine the pathogenesis of silicosis are: (1) the chemical nature and size of the dust; and, (2) the concentration and length of exposure to the dust which determines the amount inhaled.

Silica exists in three natural forms: (1) crystalline; (2) amorphous; and, (3) cryptocrystalline, which is partially crystalline and partially amorphous. Examples of cryptocrystalline forms are flint, chert, chalcedony and jasper. Amorphous forms include opal, diatomaceous earth and tripoli. Crystalline forms of silica are quartz, tridymite, cristobalite, bentonite, keatite, coesite and stishovite. The widely used crystalline silicas tridymite and cristobalite, are formed by heating quartz to extremely high temperatures. All of these three crystalline forms have the same atomic

arrangement and are interconvertible. They are often found in the refractory bricks lining high temperature furnaces (138). Typically, it is these three forms that cause the pathological condition of silicosis.

The chemical nature of the dust, the particle size, and the concentration to a very great extent determine the resulting lung damage. Size of the particulates determines whether they will reach the lung parenchyma and, when they reach the alveoli, if they will adhere to the alveolar wall. Only particles of a very small size (0.5 - 2.0 μm) have been found to gain access to the alveoli and be retained there (45). Larger particles are removed in the upper airways (55, 155). Albert and Lippman (1) reported, after an extensive study, that alveolar deposition ranges from about 80% for particles 1.3 - 2.0 μm in size to virtually zero for particles 5.8 - 7.3 μm. Deposition fell off rapidly with increasing size of particles starting at about 5 μm. In another study most particles extracted from a miner's lung were between 1 and 2 μm (88). These studies indicate that the majority of the particles that reach the lung parenchyma and are deposited in the alveoli are between 0.5 and 2.0 μm in diameter.

In addition to the particle size, the concentration of dust particles and the respiratory volume also determine whether silicosis will occur. The U.S. Public Health Service has determined that 5×10^6 particles of quartz per cubic foot of air, less than 10 μm in size, is the concentration at which a man may work his whole life without incurring injury. A concentration of 100×10^6 particles/ft^3 of air,

however, was determined by the Public Health Service to be enough quartz to cause a healthy individual to inevitably contract silicosis (138).

Particle Clearance Mechanisms

If dust particles penetrate into the air passages and the lungs, the main mechanisms involved in their clearance include: (1) the bronchial mucus sheet; (2) the alveolar phagocytic reaction; and (3) the pulmonary lymphatic system. The elimination of inhaled isotopically labelled dust particles by all three mechanisms has been studied carefully in man and animals. Each mechanism makes a relative contribution to the clearance.

The first clearance mechanism, the bronchial mucus sheet, has been found to remove 60% of the dust that is deposited. This accounts for the dust particles deposited in the nasopharynx, the trachea and the larger bronchi. The particles are rapidly removed by bronchial mucus. This mechanism, termed the "broncho-eliminative" mechanism, is actually a mucus blanket transport system which occurs as a result of mucus moving because of the whipping action of cilia. The ciliary action, which is wave-like and highly coordinated, conveys the particulate matter reaching the major bronchi back towards the trachea, epiglottis and nasopharynx where it is coughed up or swallowed.

The second clearance mechanism is the alveolar phagocytic reaction. This mechanism of clearance depends on mononuclear phagocytic cells or macrophages. Alveolar macrophages are the critical cells

in the pulmonary response to a large number of noxious environmental particles (34). Inhaled or intratracheally injected particles increase the numbers of these macrophages (10). The increased numbers of free alveolar macrophages correspondingly increase the likelihood that foreign insoluble particles will be phagocytized and removed (83). Thus, the amount of dust eliminated from the lung after dust exposure is proportional to the number of free phagocytic cells. In support of this Kavet and Brain (75) have shown that the phagocytic efficiency of the pulmonary mononuclear-phagocytic system is related to both the numbers of resident macrophages and their functional status. A depression in the macrophages' quantity or efficiency can result in a persistence of nonviable particles on the alveolar surface where such particles may exert toxic effects (75).

When there is sufficient number of resident macrophages, this mechanism, the alveolar phagocytic reaction, has been found to slowly move about 30% of the deposited particles over a period of several days. This alveolar phagocytic component untimately joins the bronchial muco-ciliary apparatus. Often these first two mechanisms of clearance, the bronchial mucus sheet and the alveolar phagocytic reaction are described as one. Gross (45), for example, has described an upper "respiratory tract component" which involves a proximally moving mucus blanket and an alveolar component which transports the inhaled dust to the pharynx.

The third clearance mechanism, the pulmonary lymphatic mechanism, is the means by which dust particles penetrating into the

interstitial tissue are removed. This mechanism is the slowest and has been found to move only the remaining 10% of the inhaled particles. As a result, the pulmonary lymphatic clearance plays a relatively minor role in the overall clearance process (121).

Lymphatic clearance depends on particle penetration. Particle penetration involves the passive movement of particles from the alveolar compartment to interstitial tissue, or to lymph vessels, without the assistance of other active processes, such as phagocytosis (16). There are three schools of thought on the subject of particulate penetration. One school suggests that only "free" particles penetrate the alveolar epithelium (134). Another school of thought suggests that the alveolar epithelial cells may round up and desquamate to become alveolar macrophages, leaving those portions of the alveolar surface uncovered. This allows free transit areas for the penetration of particles (141). The third school of thought is that the alveolar penetration of particles occurs mainly, if not exclusively, as a result of endocytosis (132). Endocytosis is the process whereby external substances (such as silica particles) are captured by an area of the plasma membrane which pinches itself off from the cell surface to form an intracellular vacuole or vesicle.

After particle penetration into the interstitial tissue the dust is transported to satellite lymph nodes via lymphatic vessels. With silica particles large deposits frequently are found in the intrapulmonary and extrapulmonary lymph nodes (82).

Besides the three principal mechanisms of particle clearance, the bronchial mucus sheet, the alveolar phagocytic reaction and the

pulmonary lymphatic system, one other minor clearance mechanism has been postulated. Godwin and Jagatic (41) have suggested that silica particles from interstitial spaces or lymphatics enter the blood system directly. Silica has been localized in the spleen and liver (138). Deposits also have been found in the peritoneal cavity of coal miners (8).

In summary, the body effectively uses several mechanisms to assist a lung exposed to foreign particles. The relative importance of these various clearance mechanisms undoubtedly is dependent on certain physico-chemical and biological characteristics, such as size, of the contaminating particles.

Theories of Silicosis

Numerous theories have been postulated to describe the mechanism of silicogenesis. These theories may be separated into three groups: physical theories, chemical theories and the immunological theory.

Physical Theories. Five different physical theories have been postulated. These include the theories of: (1) angularity; (2) valence; (3) negative charge; (4) crystallinity; and (5) surface adsorption. The first physical theory, the angularity theory, proposes that the sharp edges of quartz particles pierce and abrade pulmonary tissue until a nodular fibrosis develops (156). The second theory, the valence theory, suggests that freshly fractured silica particles have unsatisfied charges which are reactive and are responsible for biological activity (56). The negative charge theory of silicosis,

postulates that the presence of negative charges on the particle surface interacts with the positively charged protoplasm of the cell (72). The theory of crystallinity suggests that the crystal structure is responsible for silica pathogenicity (79). This latter theory was disregarded, because of the recognized fibrotic potential of amorphous silica (156). The last physical theory which has been postulated is the surface adsorption theory which depends on the adsorption of quartz particles onto other substances (such as cell membranes). This is thought to cause the development of silicosis because of the physico-chemical interaction at the quartz surface (133). However, no single physical theory sufficiently explains the disease process.

Chemical Theories. Chemical theories have also been postulated. These theories include: (1) solubility; (2) extended solubility; (3) phospholipid fibrogenic; and (4) chemical surface theory.

On the basis of his early animal experiments with silicosis, Kettle (77) was the first to support the chemical solubility theory. The silica molecule in its naturally occurring state consists of a polymerized form of large numbers of tetrahedra units represented by the formula SiO_4. These tetrahedral units are linked to each other, with each O-atom being common to two tetrahedra (68).

When quartz crystals are cleaved this results in the formation of an "active" surface on the tetrahedral complex. An incomplete monolayer of silicic acid is formed (67). Thus, free quartz particles in solution release silicic acid. What the chemical solubility theory

postulates is that once silicic acid molecules are formed they are then polymerized to colloidal dimensions. These molecules then become fixed on the protoplasm of the cell and eventually fibrosis develops (54). How this fibrosis developed was not suggested.

King (78) however found that the solubility of silica did not affect the rate and severity of tissue fibrosis. Others (11) later found no correlation between solubility and fibrogenic properties. Thus, it was concluded that the release of silicic acid from the surface of a particle is unlikely to cause the development of silicosis.

The solubility theory in its original form failed to explain the toxic properties of silica, however extensions of the original theory have been proposed.

Holt (66) attempted to modify the solubility theory of silicosis to an extended solubility theory. It was suggested that silicic acid diffuses out of phagocytes after the ingestion of silica. Silicic acid is released into the intracellular fluids and there is subsequent death of the cells because of the change in pH. The macrophages are then disrupted and there is a liberation of lipid substance. These lipid substances stimulate the formation of collagen precursors, such as mucopolysaccharides. However, this modified theory still is not universally accepted. One of the main arguments against this modified theory is that the quartz crystals located in silicotic nodules bear no relation to the size, shape and location of the collagen fibers. For this reason another chemical theory has been postulated.

Silicic acid also has been considered the main factor responsible for producing typical dense fibrotic nodules characteristic of silicosis and thus is the basis of the phospholipid fibrogenic theory. Other causes also have been suggested. Many investigators (22, 25, 36, 68) have attributed the toxicity of silica to hydrogen donation by polymeric silicic acid which forms hydrogen-bonded complexes, especially with phospholipids of the cell's phagosomal membrane. The silicic acid formed by hydrolysis of the surface of the silica combines with the molecules forming the wall of the phagosome sac, thus causing its disruption. Disruption of the phagosome releases the contained lysosomal enzymes, leading to cell death. The products of the disintegrating macrophages appear to stimulate a fibrous reaction.

Heppleston (61) summarized the cytotoxicity of silica generally as a dual surface effect involving cell membranes as well as silica particles. This is the basis for the chemical surface theory. Silicic acid, present in patches on the phagosomal membranes of silica-ingested macrophages, reacts with these membranes to produce modification of surface tension and increased permeability (111). With this increased permeability there is leakage of lysosomal enzymes and subsequent death of the cell.

Immunological Theory. A third general theory on the pathogenesis of silicosis suggests immunological cause. This theory proposes that in the specific reaction of the host to silica dust an "immunological factor" is released. This factor then stimulates fibroblasts to produce reticulin and collagen leading to the formation

of hyaline nodules and fibrous tissue. This theory was formulated because of the increased prevalence of autoimmune diseases in silicosis (124). Correlations between fibrogenic silicosis and rheumatoid arthritis have been reported (15). Vigliani et. al. (145) observed that the sera of most silicotic patients show a definite increase of gamma globulins. Since antibodies belong mostly to the gamma globulin group, they suggested that the increase was an expression of antibody production. Plasma cells of the silicotic nodules and of the regional lymph nodes were thought to be the source of gamma globulins which have been found to constitute the major part of the proteins of the silicotic nodule (17).

It has been suggested, if silicosis has an immunological pathogenesis, that silica dust may stimulate the formation of new antigens and, subsequently, of antibodies to these antigens (146). Alternatively, silica could act (1) as a stimulator for the formation of autoantigens; or (2) as a booster of heteroantigens. Also, it has been postulated that silica may liberate autoantigens indirectly by killing a comparatively large number of cells and exposing chemical substances not normally seen by the immune system (156).

If silica has an immunological pathogenesis, then it is of interest to understand how this particulate affects a host's immune responsiveness. One of the aims of this study was to investigate the long term effects of silica on the immune responsiveness to an antigen.

Effect of Silica Dust on the Immune Response

Nonspecific effects of silica dust on the immune response have been reported. Antibodies have been produced when quartz was injected by various routes (147). Pernis and Paronetto (119) were able to produce a nonspecific stimulation of the immune response by the injection of rabbits and rats with crystalline silica. They found an enhanced antibody response in these animals to protein antigens and suggested that the adjuvant effect of silica is related to the proliferation of immunologically competent cells in the reticuloendothelial system.

Amorphous silica has been shown to have an adjuvant effect in guinea pigs when injected subcutaneously together with bovine gamma globulin (96). Amorphous silica also enhanced the immune response to bacillus Calmette Guerin (BCG) and to both particulate and soluble antigens. Antigen absorption onto the silica particles was not necessary for its adjuvant activity, but antibody appeared earlier and persisted longer when antigen was injected simultaneously with silica. When crystalline silica was used, a more pronounced adjuvant effect was found in rats who received the antigen some time after silica injection (96). Using ovalbumin absorbed to bentonite particles (a crystalline silica) to inject rabbits, Wilkinson and White (153) found a high serum antibody titer. They explained this adjuvant effect by indicating that ovalbumin absorbed to bentonite remains at the site of injection for a longer period of time than ovalbumin in solution. Using quartz particles in incomplete Freund's adjuvant,

high primary and secondary antibody responses were produced against bovine serum albumin (BSA). When mice were immunized with BSA absorbed to bentonite, the antibody responses elicited were even higher. It was suggested that the capacity of this silica to act as an adjuvant is related to its ability to increase lysosomal membrane permeability, by reacting with the surface of the lysosomes (139).

More recent studies have demonstrated effects of silica dust that suggest changes in cell-mediated immune function. Prolongation of skin allografts occurred after intraperitoneal injection of silica dust (117, 118). Also, the ability of silica to abrogate hybrid and allogeneic resistance to bone marrow grafts has been shown (91).

The effects of particulate silica on the bactericidal action of the pulmonary protective mechanisms have been contradictory. Increased bactericidal action in vivo and in vitro has been found, using macrophages from mice intratracheally injected with silica (43, 44). In a recent investigation, silica intravenous injection has been found to enhance susceptibility to gram-negative infection, probably by destruction of macrophages (32). A significantly decreased phagocytic index of alveolar macrophages has been reported by Miller and Zarkower (102) when silica was introduced by inhalation.

Levy and Wheelock (90) attempted to determine whether the cytotoxic effects of silica in vivo were due solely to destruction of macrophages or were the result of direct or indirect effects of silica on other cell types. They indicated that silica had a direct

depressive effect on cells other than macrophages. Indirect effects on lymphocytes were reported, most likely produced by "factors released from silica-lysed macrophages." A survey of different preparations of silica revealed a great variation in their immunodepressive properties.

Miller and Zarkower (102) have demonstrated an immunosuppressive effect of inhaled silica dust on the response of B lymphocytes to aerosol-administered Escherichia coli lipopolysaccharide (LPS). When LPS was administered by an intravenous route suppression was less, but still statistically significant. Employing LPS as a B lymphocyte mitogen in vitro, they showed a decreased uptake of ^{3}H-thymidine in spleen and mediastinal lymph node cells from silica-exposed animals. However, an enhanced responsiveness of mediastinal lymph node cells was found when concanavalin A (Con A), a T cell mitogen, was used. An enhancing effect also was seen in the in vitro response of spleen lymphocytes from silica-exposed tuberculin-sensitized animals to purified protein derivative (PPD) of tuberculin. In addition, they reported that alveolar macrophages from silica-dusted animals had less ability to phagocytize E. coli in vitro. Upon transfer of these in vitro antigen "fed" macrophages to normal syngeneic recipients, both spleen and alveolar macrophages from exposed animals exhibited decreased capacity to phagocytize antigen and initiate antibody formation. Thus, short term inhalation of silica dust changes the antigen and mitogen reactivity of T and B lymphocytes and damages the macrophage-antigen processing system.

Later experiments by Miller (101) and Miller and Zarkower (104) employing silica particles suspensions and supernatant fluids from these suspensions determined the liberation of a soluble, dialyzable toxic factor. This factor was found to induce suppression of the B cell response to soluble LPS and to enhance the reactivity of T cells in the lymphocyte-mediated cytotoxicity response. A similar suppression was seen using commercially prepared silicic acid. Miller (101) proposed a mechanism whereby silica particles and/or liberated toxic factors may act to alter immunoglobulin receptors on B cells and thereby inhibit antigen binding and activation of the immune response. They also may act on T cells by uncovering antigen binding receptors allowing enhancement of their activity.

Over short periods of silica exposure it has been conclusively demonstrated experimentally that high concentrations of silica dust do have the capacity to seriously affect tissues involved in immunological responses. Short term exposures have been shown to damage critically the macrophage antigen-processing system and to change the mitogen and antigen reactivity of T and B lymphocyte populations. In contrast, it was the purpose of this study to investigate, both immunologically and histologically, how the cells and tissues of the murine model are affected by long term exposure to silica dust. Some defined immunological parameters for long term dust exposures on the mouse were evaluated and correlated with the chronic pathological picture as seen by light microscopy and scanning electron microscopy.

Pathogenesis of Silicosis

Silicosis results in the formation of granulomas which become progressively more fibrotic. The term granuloma implies a small 1 to 2 mm collection of macrophages or histiocytes almost invariably surrounded by a rim of mononuclear cells, principally lymphocytes (123). Granulomas eventually are converted into an acellular nodule consisting of concentric layers of hyaline-appearing connective tissue (159). The events in the pathogenesis of silicosis have been extensively studied (63, 98). Silica particles are inhaled and retained in the lung periphery where they are phagocytized by macrophages. From this location a large percentage of the ingested particles are carried by the lymphatics to the lymph follicles in peribronchiolar positions in the lung. However, some particle-containing macrophages become fixed in lymphoid tissue adjacent to terminal bronchioles. Death of these macrophages occurs with subsequent release of their cellular contents, including the ingested silica particles. This stimulates the aggregation of more macrophages, which are also killed, releasing their contents and in some way causing the recruitment of other cells such as reticulin cells and fibroblasts. Reticulin fibers are rapidly formed. Subsequently, there is a conversion of the reticulin to dense collagen and the development of fibrotic nodules. Continuous exposure to silica causes these nodules to gradually increase in size. Complications arise when the nodules coalesce and streamers of fibrous tissue distort and compress blood vessels and pulmonary tissue. Heppleston (60) summarized the events in the pathogenesis of the silicotic nodule

as including four basic processes: (1) necrosis of macrophages after silica ingestion and then liberation of the dust; (2) attraction of new macrophages to rephagocytize the free particles; (3) collagen formation; and (4) hyalinization. Eventually the lung parenchyma is grossly altered. Increasing resistance to pulmonary blood flow with the concurrent development of numerous foci of emphysema occurs.

Perifocal emphysema may be defined as a focal dilatation of air spaces with accompanying destruction of their walls involving part or whole pulmonary lobules adjacent to areas of persistent atelectasis or scarring. The scarring and atelectasis collapse alveoli and thereby increase the elastic pull on adjacent alveoli. The vascular supply to the distended air spaces is imparied by either distortion or damage to the microcirculation. The small respiratory bronchioles are similarly narrowed. With such bronchiolar narrowing, air trapping may contribute to the distension of alveoli, and, in turn, the distension further impedes blood supply (123). In this way focal areas of the lung become emphysematous. In silicosis, this combination of nodule formation and emphysema is responsible for the pulmonary insufficiency which is characteristic of the disease.

The main purpose of this investigation was to study the histological changes caused by silica exposure and the dispersion of inhaled silica to various tissues, using light microscopy, scanning electron microscopy and x-ray analysis. In reviewing the literature, it is apparent that the overall classical tissue reaction of the lung to the deposition of airborne silica is the formation of discrete nodules (138). In the naturally occurring disease in man, three

distinct types of tissue reactions have been described: the chronic, the accelerated and the diffuse type reactions. The chronic tissue reaction is observed when there is a moderate exposure of dust extending for a period of 20 to 40 years. When there is a larger particle dosage for a period of 5 to 15 years, the tissue reaction is termed accelerated. And finally, when there is a very heavy alveolar deposition of particles for a period of less than 5 years, the tissue reaction may be described as diffuse (159).

The chronic type of tissue reaction, which produces the classical lesion, the silicotic hyaline nodule, usually is observed in people working in industries where the quartz percentage in respirable dust is less than 30%. It has been reported (46) that when the percentage of crystalline silica exceeds 18%, the tissue reaction evoked is silicosis. In the chronic silica-caused disease three separate layers in the pulmonary silicotic nodules usually are described: (1) the hyaline core; (2) a connective tissue layer; and (3) an irregularly dispersed connective tissue layer. The core of the nodule consists of a dust-filled hyaline zone. This core is surrounded by a concentric zone of cellular connective tissue that contains no particles. It is in turn, surrounded by a zone of irregularly dispersed connective tissue which also contains crystalline silica. It is from this last irregularly dispersed connective tissue zone that silica is carried to enlarge the nodules and to establish new nodules in other areas of the lung (138).

In man it takes approximately 20 years of dust exposure to produce silicotic nodules. In recent years experiments on silicosis

have been designed where the dust concentration of the most severe industrial conditions is simulated. Under these conditions silicotic lesions can be produced experimentally (144). A number of animal models have been used and they have provided useful information regarding the development of the silicotic lesion.

Experimental Studies

The selection of the animal species for experimental silicosis is of prime importance. Between species there are wide genetic differences, minor variations in the anatomy of pulmonary tissue, as well as various endocrine influences and enzymatic differences.

Previous immune investigations have established the mouse as an excellent model system to measure changes in immune responsiveness after particulate exposure (103, 158). For this study, the immunologically-defined murine model was used. The purpose of this investigation was to combine long term immunological studies with histopathological investigations of the developing silicotic lesion and the localization of silica dust in the tissues.

Silicosis similar to that seen in man has been produced in a number of animal species; rats (79), rabbits (35), guinea pigs (35), and monkeys (159). Rats and guinea pigs supply the closest comparison to our animal model.

Rats. One of the most common laboratory animals employed in experimental silicosis is the rat. Rats have been exposed to silica dust at a concentration of 30,000 particles/ml (less than 5 μm in diameter), for 18 hours a day, five days a week, for a period of one

year (156). This is roughly sixty times the exposure used in our studies (particles size averaging 2 μm). King et. al. (80) found thickening of the alveolar wall in the first few months of dust exposure. The hilar lymph nodes were found to have a slight increase in the amount of reticulin. By 220 days there were collections of dust-laden macrophages adjacent to small blood vessels in the lung. Collagen was detected in hilar nodes at this time. By 200 days the rat lungs were observed to be composed of typical collagenous silicotic nodules. Others (152) have produced typical round compact granulomas in rat lungs after 22 months exposure in dust chambers. These rats inhaled a dust concentration of 45 mg/m^3 for five hours a day, five days a week. This is approximately ten times the concentration of our study of 4.9 mg/m^3 for 24 hours a day, up to 39 weeks.

Guinea Pigs. Guinea pigs were exposed to quartz dust at a concentration of 4000×10^6 particles/$ft.^3$ of air, eight hours a day for five days a week, over a period of two years (156). This is approximately ten times the exposure used in our study. After inhalation of quartz in guinea pigs, Gardner (36) observed that dust particles which were phagocytized by macrophages, eventually killed the macrophages. Particles were liberated and again phagocytized by recruited macrophages. Collections of quartz-laden phagocytes were found adjacent to blood vessels. After a few months cellular proliferation and a laying down of fibrous tissue was seen. At this time an area also developed which had numerous giant cells. Fibrous silicotic collagenous nodules finally developed after two years.

Differences Between Human and Experimental Silicotic Lesions

The human silicotic lesion in general resembles the experimentally-produced lesion in morphology and distribution. The experimentally-produced lesions differ from human lesions in that they lack a capsule. These nodules also are smaller than human lesions, and they have fine loose fibers. Experimental lesions are less hyalinized and the hyaline is arranged in a network. Human lesions arise from interstitial tissue but animal lesions arise from the intra-alveolar structure (156).

Use of Microscopy to Evaluate the Progression of the Silicotic Lesion

All of the early studies employed light microscopy to evaluate the progression of silicotic lesions. The first stages in the development of a silicotic lesion in the rat lung were observed by Gross et. al. (52). Intra-alveolar aggregations of dust-laden phagocytes were found bound together by an intercellular framework of reticulin fibers. The mature silicotic lesions were observed under light microscopy both in man and animals (52). Lesions were found in the walls of alveoli arising from respiratory bronchioles and alveolar ducts. Nodules also were found formed around the branches of the pulmonary arteries. This led to obliteration of perivascular lymphatic channels and to eventual destruction of the arterial wall.

Although much was derived from examination of histological sections under light microscopy, it was necessary to correctly evaluate cellular changes at the level observable by electron microscopy. Investigators (122) examined intraperitoneal silicotic nodules in rats.

Reticulin fibrils, intra- and extracellular silica particles were observed. However, these findings of silicotic lesions were not representative of silicotic lung lesions. Difficulty in the preparation of silica-exposed tissue hindered subsequent investigations of cellular changes using transmission electron microscopy.

Recently the scanning electron microscope (SEM) has become an invaluable tool in examining the ultrastructural morphology of pulmonary tissue. Its ability to examine a large surface area rapidly, at either high or low magnification, and its great depth of focus, make it extremely useful in the study of cellular changes after silica exposure.

However, the first reported attempts (65, 73) to use SEM to study the lung were hampered by lack of proper methods of lung tissue preparation. Shrinkage and distortion often resulted when tissues were fixed with fluid fixatives. These tissues were either dried in a vacuum, dehydrated in successive grades of alcohol (or acetone), or air-dried (120). Wang and Thurlbeck (150) however, did report satisfactory results with this air-dried method. Nowell and Tyler (113, 114, 115) compared a variety of methods of preparing pulmonary tissues and established that tissues prepared by aldehyde fixation followed by drying with the critical point method of Anderson (6) were most satisfactory. This method, which was used in this investigation, involves bringing tissues from a wet to dry condition without the distorting effects of surface tension during drying. The critical point is the combination of critical temperature and critical

pressure. A critical temperature is reached when the liquid which is heated in a confined space, is completely converted to gas. When the pressure in the confined space increases, the density of the vapor phase of the liquid increases. The critical pressure is when the density of the vapor phase equals the density of the liquid phase. Tissue carried through the combination of critical temperature and critical pressure (the critical point) are brought to dryness without having passed through the surface of the membrane (18). Thus, there are no distorting effects of surface tension during tissue dehydration with this method.

Using the critical point method to prepare tissues allowed a very comprehensive investigation of morphological changes by the SEM. These observations of lung tissue under SEM, in conjunction with the histological changes seen under light microscopy, permitted evaluations of the progression of silicotic lesions over a period of 39 weeks.

Use of Microscopy to Localize Silica in Tissue

An important step towards understanding the mechanism of silicogenesis is identification of silica dust within tissues. Distribution of silica within silicotic lesions has been investigated under light microscopy both in man and in rats (51, 52). The method of microincineration combined with photomicrography using dark-field illumination has been used (53). Another method, polarized light microscopy, illuminates birefringent particles of silica that can be localized in particular histological areas. The amount of demonstrable silica is usually very variable (23). Most of the silica is found in

the outer layers of the nodules and in interstitial extensions of the silicotic lesion. Some silica may be found free in the surrounding air spaces of the lung, and silica flocs can be demonstrated adherent to the alveolar walls, which show no structural damage (57).

Recently a new technique, energy-dispersive x-ray analysis (EDXA), has been used in the identification of silica dust in tissue. EDXA is an analytical technique which allows simultaneous multi-elemental analysis while the specimen is observed under SEM (154). Using this technique, Funahashi et. al. (33) established the levels of silica dust in normal and silica-exposed human lung tissue. Morgenroth et. al. (106) used EDXA and SEM to identify silica in guinea pig lung macrophages. These macrophages were embedded in Epon and thin-sectioned. Others (125) have used specimens from ordinary paraffin blocks to demonstrate the feasibility of EDXA and SEM in the identification of silica deposited in lung tissue.

One purpose of this investigation has been to localized silica in dust-exposed tissue. Using polarized light microscopy permitted visualization of silica dust at its location within the tissue. This allowed a detailed study of the site of deposition in relation to the tissue reaction observed. EDXA indicated the amount of dust that could be localized after very short exposure intervals to the silica aerosol. In addition, EDXA identified sites of deposition too small to be detected under ordinary light microscopy.

In summary, this study attempted to investigate the mechanism of silicogenic action on cells and tissues through: (1) producing the

chronic pathological condition of silicosis in an animal model; (2) evaluating the capacity of chronic silica-exposed mice to mount an immune response; (3) observing the progression of the silicotic lesion by means of light microscopy and scanning electron microscopy; and (4) localizing silica in cells and tissues by means of polariscopic light microscopy and energy-dispersive x-ray analysis.

In order to produce silicotic lesions, it is necessary to understand the many factors which determine the development of this disease. One factor is the efficiency of pulmonary particle clearance. Another important factor is the mechanism of how silica causes disease, i.e., theories of silicogenesis.

This investigation demonstrated the compromised immune response of silica-exposed animals over a period of time. This investigation also demonstrated the progression of the lesion and the localization of silica dust in cells and tissues over this same time period. The following methodology was used to demonstrate these effects.

MATERIALS AND METHODS

Animals

Inbred, female, Balb/c mice weighing between 15 and 25 grams were obtained from Flow Laboratories. These animals were housed in plastic cages and allowed commercial mouse pellets (Purina Laboratory chow) and water ad libitum.

Silica Dust

The silica dust used was Min-u-sil (The Pennsylvania Glass Sand Co., Pittsburgh, Pa., lot #76824-A), a crystalline form less the 5 microns in size.

Silica Dust Exposure Facilities

Exposure chambers of stainless steel and glass designed by Hinners et. al. (64) were used to house the mice. Appropriate environmental temperature (72^{o} C) and relative humidity (50%) were maintained throughout the experimental time period. The incoming air was continuously filtered by a chemical, biological and radiological filter and flowed at a rate of 5 ft^3/min. A high velocity jet of air was pulsed into silica dust lying at the bottom of a generator (21) creating the aerosol. The length of time between pulses and the individual length of each pulse were controlled by relays that activated a normally closed solenoid valve. These relays in turn could, by nature of their timing, regulate the dust concentration in each chamber. In addition to concentration, particle size of the silica dust was

critical. For this reason a cyclone particle fractionator was included in the system to assure a minimum size distribution for particles being generated into the chambers.

Sequential Sampler

The concentrations of silica dust at all times were monitored by a Sequential Sampler Model B (Research Appliance Corporation, Pittsburgh, Pa.). The instrument filtered air passing from the exposure chamber for designated time intervals (every other two hours for 48 hours). The filters were weighed and the amount of silica dust in a measured volume of air over time was calculated. The mass median diameter of the silica dust entering the chambers was 2.0 μm.

Animal Sacrifice and Organ Removal for Immune Studies

Mice were anesthetized with ether and sacrificed by decapitation. Trunk blood was collected from each animal for serological study. Animals were then dipped in 70% ethanol and the spleens, lungs and mediastinal lymph nodes removed. Spleen and mediastinal nodes were placed in Hank's Balanced Salt Solution (HBSS). Lungs were emersed in 10% phosphate buffered formalin for histological observation.

Antigen Preparation

Escherichia coli Antigen. E. coli 055:K59:NM (055:B5) was the antigen employed for aerosol immunization procedures. The organism was cultivated in trypticase soy broth (Difco Laboratories, Detroit, MI.) at 37° C for 72 hours. The bacteria were then harvested by continuous flow centrifugation. After being washed in saline, ethyl

alcohol and acetone, the bacterial were dried at 37° C. To produce a fine powder the dried cells were ground with a mortar and pestle. The cells were suspended in saline at the proper concentration and the suspension was dispersed by ultrasound and autoclaved prior to use.

Sheep Red Blood Cell (SRBC) Antigen. The single source of all red blood cells was a sheep maintained by The Pennsylvania State University, Department of Veterinary Science. Blood was collected and stored in Alsever's solution for a limited period of time before use. The blood was washed in sterile HBSS and diluted to the proper concentration before use as an antigen.

Lipopolysaccharide (LPS)-Coated SRBC Antigen. SRBCs were coated with E. coli 055:B5 LPS for use in the Jerne immunoplaque assay and hemagglutination titrations (31, 105). LPS in saline at a concentration of 1.0 mg/ml was boiled for one hour. One part packed, washed SRBCs were added to nine parts LPS solution and incubated for one hour at 37° C. The LPS coated SRBCs were washed three times in saline before use and diluted to the appropriate concentration.

Immunization Procedures

Aerosol was the major form of immunization employed in this investigation. A Model A 4 Airborne Infection Apparatus (Tri-R Instruments, Inc., Rockville Center, N.Y.) designed by Middlebrook (99) was used. The air flow settings were 190 on the Main Air Flowmeter (47.3 liters/min.) and 120 on the Compressed Air Flowmeter (5.3 liters/min.). Ten ml of a 50 mg/ml concentration were dispersed as an aerosol for one hour.

Intravenous immunization was done by injecting mice with 0.5 ml of the antigen into the lateral tail vein. The antigens were: a solution of whole, heat-killed E. coli 055:B5 at 100 µg/ml and a suspension of SRBCs at 4×10^8/ml.

Immunoplaque Assay

Dresser and Wortis' (24) modification of the Jerne immunoplaque assay was used to measure the IgM-forming capacity of the immunized animal. After placing the spleen or mediastinal lymph nodes in cold HBSS, single cell suspensions were made by teasing the whole organ with forceps through a 60 mesh steel screen. A 200 mesh screen was then used to disrupt large clumps and filter out connective tissue. The cells were centrifuged at 400 x g for 10 min at 4° C and the supernatant was poured off. The cells were then resuspended in HBSS to an appropriate concentration. Cell counts were done on a Coulter Counter Model B (Coulter Electronic, Inc., Hialeah, Florida). The appropriate amount (0.1 ml) of lymphoid cells was added to a 1.0 ml volume of LPS-coated SRBCs. The cells were mixed and poured into a 60 x 15 mm glass petri dish having a 3.0 ml base layer of 1.2% agarose in HBSS. After the agar solidified the plates were incubated for one hour at 37° C. Absorbed guinea pig complement (1.5 ml) diluted 1:15 in HBSS was then added to each plate and the plates reincubated. After one hour the complement was removed. The number of plaque-forming cells (PFC) was determined in each assay by establishing the number of hemolytic plaques using a stereoscopic microscope and a Sensaur Bacti

Counter (Accu-Tech Corporation, New York, N.Y.). The number of PFC was expressed as plaques/organ and plaques/10^6 nucleated cells.

Hemagglutination Titers

Hemagglutination titers, using the microtiter system (Cooke Engineering Company, Alexandria, Va.) were established to determine the serum antibody activities. Serial dilutions were made starting with 0.025 ml heat-inactivated (56° C for 30 min) serum samples. In addition 0.025 ml LPS-coated SRBCs or SRBCs in a 0.5% solution were placed into each well. The plates were then sealed with plastic, gently mixed and incubated for one hour at 37° C and refrigerated overnight at 4° C. The reciprocal of the highest dilution of serum exhibiting complete agglutination was used as the titer for each animal. For statistical analysis the values obtained were converted to $\log_{10}$ (92).

Statistical Analysis

The mean values for each group were compared and analysis of variance (F-distribution test) was performed (140). Differences between means were considered significant when $p < 0.05$.

Animal Sacrifice, Organ Removal and Histological Procedures

The animals were sacrificed after a sublethal intraperitoneal injection of sodium pentobarbital. The thoracic cavity was opened and the lungs were perfused intravascularly (37 - 40) *in situ* with 0.85% saline. The lungs were next perfused with cold cacodylate buffered formaldehyde-glutaraldehyde (Karnovsky's fixative) (74) diluted with

1 part distilled water and 3.5 parts of 0.2 M cacodylate buffer (pH 7.2). This fixative was necessary because the entire left lobe of the lung was used for scanning electron microscopy. The heart, trachea and lungs were then removed en bloc. The right lobe was then rinsed and placed in 10% phosphate buffered formalin for 48 hours, dehydrated with ethanol and embedded in paraplast. Six micron sections were cut and stained with hematoxylin and eosin. Sections showing particular pathology were selected and stained with Masson's trichrome stain for collagen and reticulin. To detect silica particles in the tissues polarizing lenses were used taking advantage of silica's light-polarizing properties.

Animal Sacrifice, Organ Removal and Tissue Preparation for Scanning Electron Microscopy

Vacular perfusion of fixative into the lungs of heavily anesthetized female Balb/c mice was done as described above. After lung removal the left lobes were cut in half sagitally and placed in an aliquot of fixative for five hours at room temperature. The tissue was then washed for 3-12 hours in 0.1 M cold cacodylate buffer (pH 7.4). Tissue lobes were dehydrated through graded ethanol and dried by the critical point method of Anderson (6) using Freon 113.

Coating of Specimens and Scanning Electron Microscopy

Critically-point dried tissues to be viewed for surface morphology were cemented to aluminum studs, and then were coated with gold palladium in a vacuum evaporator to a thickness of approximately 150 Å. A Mini-SEM (ISI Model 7) was used for examination and photography of lung tissue.

Preparation of Tissues for X-ray Analysis

Sections of lung tissue 6 microns thick were cut from the paraffin blocks prepared for light microscope observations. These sections were floated from distilled water onto the surface of carbon planchets (Ernest Fullam Inc., Schenectady, N.Y.) and air-dried at room temperature. To reduce electrical charging of the specimen during examinations in the scanning electron microscope, each planchet was given a 100 Å carbon coating by vapor deposition.

SEM Examination and X-ray Analysis

The lung tissue mounted on a carbon planchet was placed in the scanning electron microscope (SEM) (JEOL Model 50 A) and examined directly in the secondary electron mode for tissue landmarks such as respiratory bronchioles or terminal bronchioles. A number of signals are generated when the primary electron beam hits the specimen. Electrons in the specimen which are excited by collisions with the primary beam are termed secondary electrons. These low energy secondary electrons which escape from the specimen surface are collected and converted into a high resolution image of the specimen.

Besides secondary electrons, x-rays also are emitted when the incident primary electron beam of the SEM collides with the specimen. These x-rays generated are collected and channeled to emit a small electrical signal. This electrical signal has a charge which is proportional to the energy of the incoming x-ray. The signal is then electronically processed and stored in a computer as a single count. A spectrum is produced as incoming x-rays are detected, sorted and

stored. The x-axis of this spectrum represents an x-ray energy distribution and the y-axis represents the number of x-rays of a given energy. A cathode ray screen illuminates this spectrum and each peak visualized represents an element found in the tissue. Ten randomly selected areas of approximately 1 mm^2 were x-ray analyzed (Kevex Energy Dispersive X-ray Analyzer) to obtain the relative concentration of silica dust in the tissue.

RESULTS

Effect of Chronic Silica Dust Inhalation on the Immune Response to Whole E. coli 055:B5 Administered by Aerosol

The mice were maintained in exposure chambers for 3, 9, 15, 21, 27, 33, and 39 weeks and exposed to silica at 4932.25 ± 235.4 $\mu g/m^3$ of air. Control mice were maintained in identical chambers but were breathing only filtered air. Groups of six mice were removed from each chamber at 3, 9, 15, 21, and 27 weeks and were stimulated with whole E. coli antigen given as an aerosol.

The numbers of antibody-forming cells were determined in the spleens, and E. coli LPS agglutinating activities were measured in the sera at all time points. The numbers of antibody-forming cells were also determined in pooled mediastinal lymph nodes (MLN) after 9, 15, 21, and 27 weeks of silica exposure.

Silica exposure reduced greatly the ability of the animals in general, and the spleens in particular, to respond to the aerosolized E. coli antigen. As shown in Table 1 and Figure 1, the numbers of antibody-forming cells found in the spleens of silica-treated animals were only 14, 11, 4, 2, and 2 percent of control animals after 3, 9, 15, 21, and 27 weeks of exposure. The hemagglutination titers in the sera of the silica-treated animals were also decreased to the same degree as the responses in the spleens.

Silica exposure produced a different pattern of effects on antibody-forming activity in the mediastinal lymph nodes. The silica-treated animals had an elevated number of anti-E. coli forming

Table 1. Effect of Silica Dust Inhalation on the Immune Response to Aerosol Immunization with Whole *E. coli* 055:B5.[a]

Exposure to Silica Dust (Weeks)	SPLEEN PFC/Organ (% Control)	SPLEEN PFC/10^6 (% Control)	MLN[b] PFC/Organ (% Control)	MLN[b] PFC/10^6 (% Control)	HA Titer (% Control)
3- Control	11,991 ± 2,483	104.8 ± 18.3	ND[d]	ND[d]	3,383.76 ± .23
Silica	1,675 ± 405[c]	15.2 ± 3.5[c]			330.75 ± .20[c]
	(13.97)	(14.51)			(9.77)
9- Control	12,720 ± 2,494	106.1 ± 16.2	670	148.8	3,619.10 ± .20
Silica	1,380 ± 423[c]	10.8 ± 2.2[c]	1,930[c]	250.7[c]	193.24 ± .13[c]
	(10.80)	(10.20)	(288.1)	(168.5)	(5.34)
15- Control	21,691 ± 4,403	140.4 ± 21.6	3,110	956.92	3,225.52 ± .23
Silica	863 ± 161[c]	5.0 ± .62[c]	2,300	764.12	106.98 ± .13[c]
	(3.98)	(3.53)	(73.95)	(79.85)	(3.32)
21- Control	41,783 ± 9,593	267.1 ± 53.3	2,010	1,313.7	2,558.59 ± .14
Silica	933 ± 270[c]	6.1 ± 1.5[c]	990	515.54	134.28 ± .09[c]
	(2.23)	(2.26)	(44.27)	(39.24)	(5.25)
27- Control	17,492 ± 3,248	146.4 ± 27.9	1,635	530.84	2,737.79 ± .12
	381 ± 123[c]	2.5 ± .91	855	165.70	238.45 ± .12[c]
	(2.18)	(1.72)	(52.29)	(31.21)	(8.71)

[a] Values are expressed as mean ± S.E.M. for six mice.
[b] MLN were pooled for each group.
[c] Significant $p<0.005$ when compared to control group.
[d] Not done.

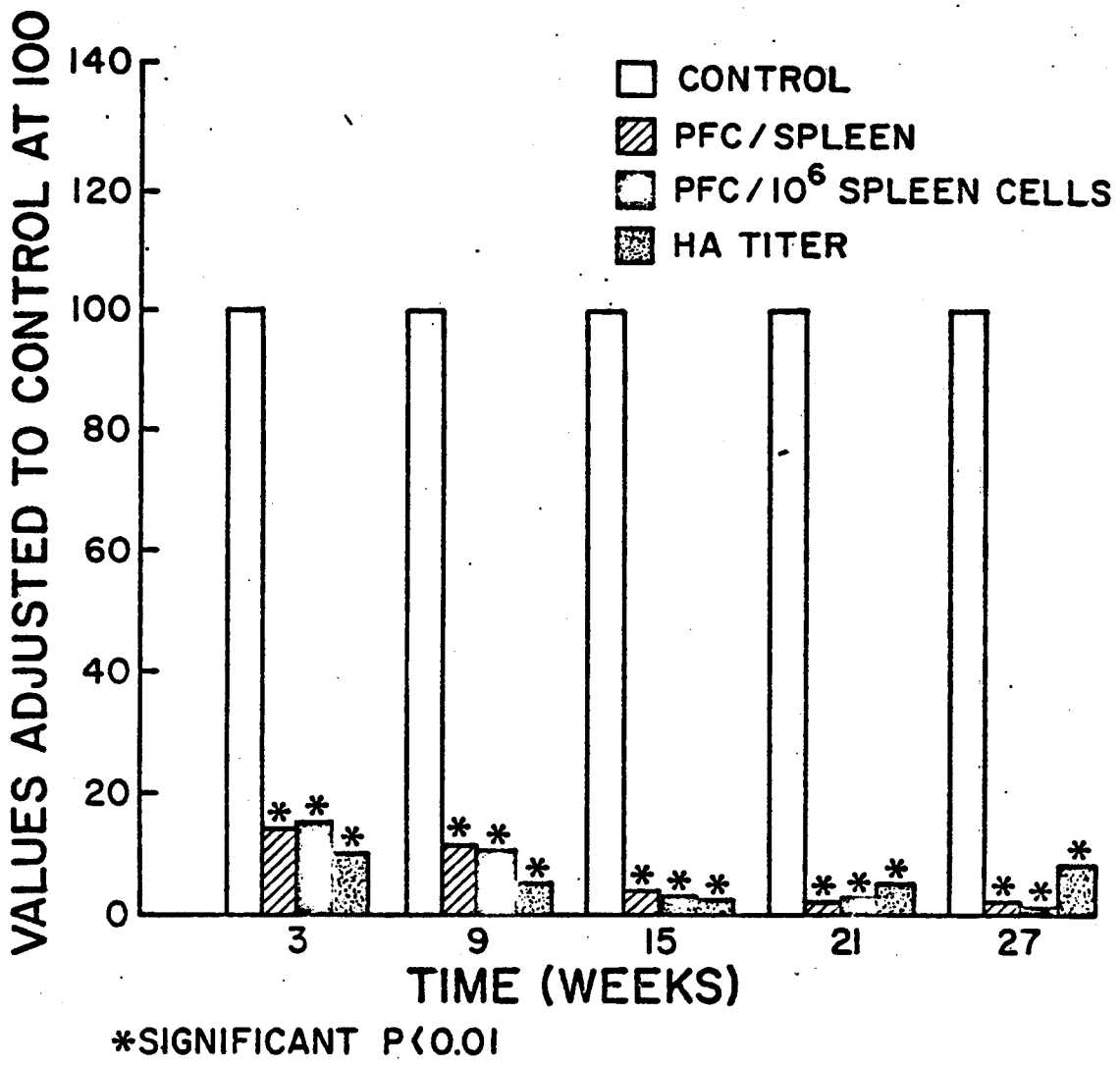

Figure 1. Response of silica exposed mice to E. coli aerosol.

antibody-producing cells after nine weeks. However, depression of response was present after 15, 21, and 27 weeks which was found to be 74, 44, and 52% of time-matched controls. These results are shown in Table 1 and Figure 2.

Effect of Chronic Silica Dust Inhalation on the Immune Response to Whole E. coli 055:B5 Antigen Administered by Intravenous Injection (I.V.)

The effect of silica dust inhalation on the immune response of mice to i.v. injected E. coli 055:B5 was next determined. Six control and six silica-exposed mice (39 weeks at a mean concentration of 4932.25 ± 235.4 $\mu g/m^3$) were injected i.v. with 50 µg of whole E. coli and assayed five days postimmunization. As shown in Table 2, mice exposed to silica dust exhibited no significant difference in their response to E. coli administered by the i.v. route in terms of PFC/spleen (111.73%), PFC/10^6 spleen cells (92.68%) and serum HA titers (95.20%) (Figure 3). However, PFC/organ and PFC/10^6 of pooled MLN were found to be greatly enhanced (368.67% and 541.16% of the controls, respectively) (Figure 4) in their ability to respond after intravenous immunization with whole E. coli. The immunosuppression of the B cell response to E. coli 055:B5 found in aerosol immunization was not seen when the silica-stressed lungs were bypassed by i.v. injection of antigen.

Effect of Silica Dust Inhalation on the Body and Spleen Weights of Mice

To determine if silica inhalation had any effect on spleen size the live mice and later their excised spleens were weighed. This was done after 3, 9, 15, 21, and 27 weeks of exposure to silica and

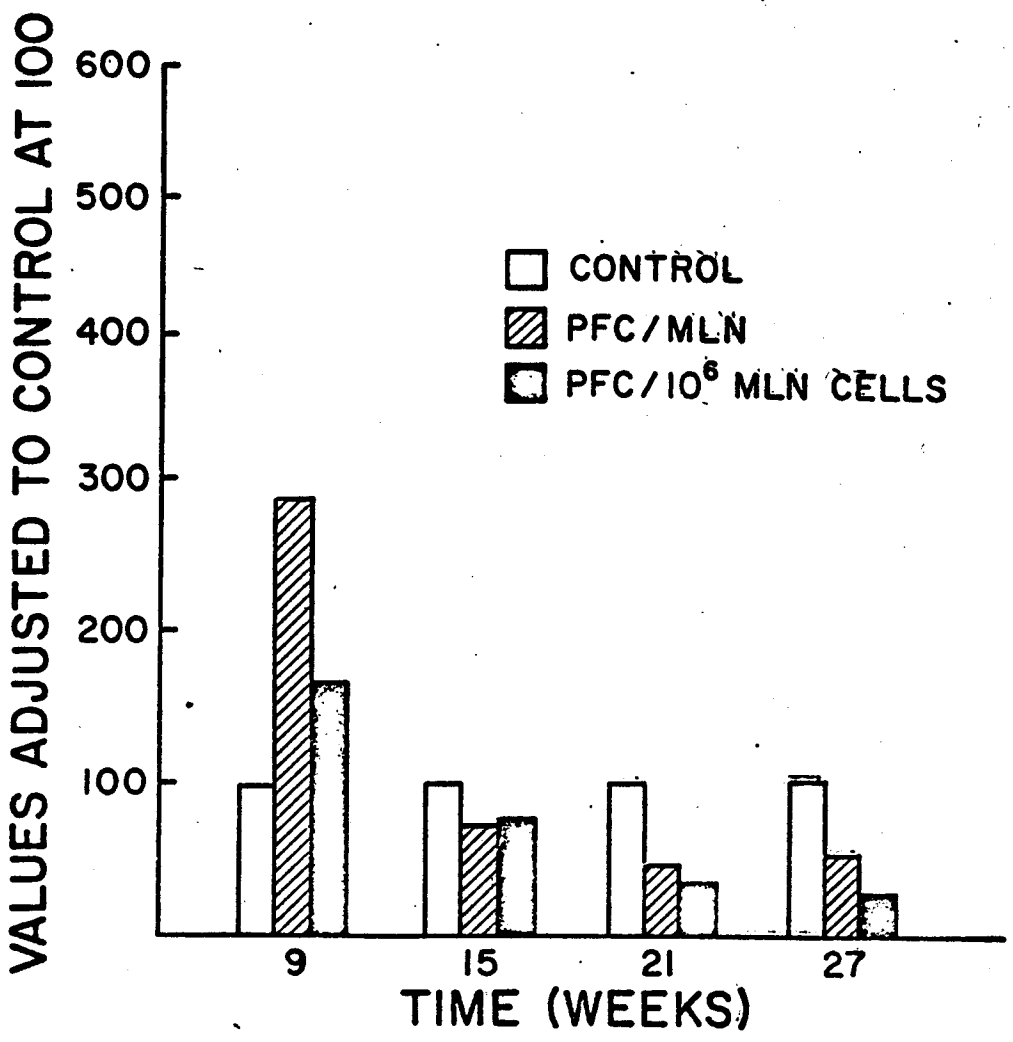

Figure 2. Response of silica exposed mice to E. coli aerosol.

Table 2. Effect of Silica Dust Inhalation on the Immune Response to Intravenous Injection with Whole *E. coli* 055:B5.[a]

Exposure to Silica Dust (Weeks)	SPLEEN PFC/Organ (% Control)	SPLEEN PFC/10^6 (% Control)	MLN[b] PFC/Organ (% Control)	MLN[b] PFC/10^6 (% Control)	HA Titer (% Control)
39- Control	67,483 ± 14,024	446.1 ± 67.6	415	70.33	950.60 ± .24
Silica	75,400 ± 18,764 (111.73)	413.4 ± 71.5 (92.68)	1,530 (368.67)	380.60 (541.16)	904.97 ± .14 (95.20)

[a] Values are expressed as mean ± S. E. M. for six mice.
[b] MLN were pooled for each group.

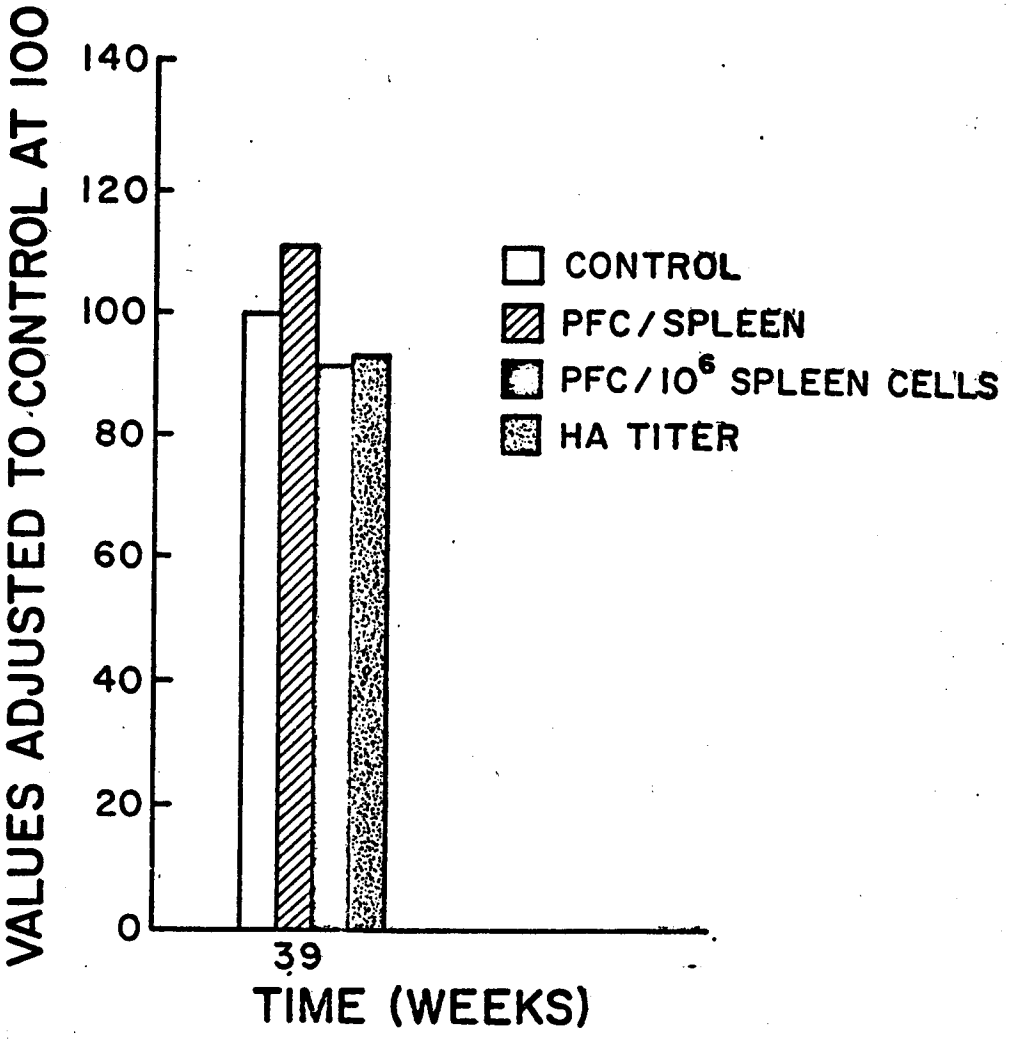

Figure 3. Response of silica exposed mice to E. coli I.V. injections.

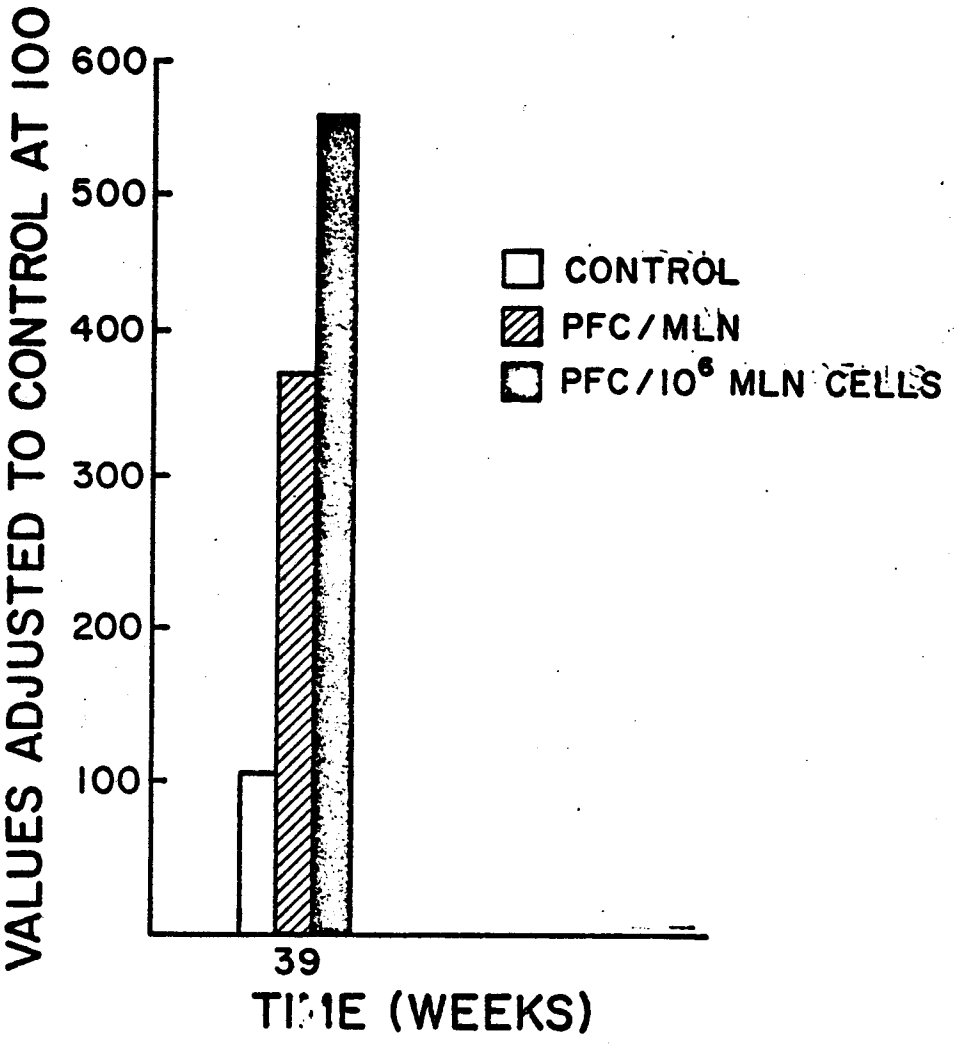

Figure 4. Response of silica exposed mice to E. coli I.V. injections.

weights were expressed as spleen/body weight ratios. These ratios were compared to the ratios of air-only exposed control animals. Individual body and spleen weights of mice were therefore taken at each exposure interval (Table 3). Body weights in general increased until 27 weeks (Figure 5), with control mice being significantly heavier ($p < 0.05$) at 27 weeks of exposure. Spleen weights were found to increase gradually in the silica-exposed animals and remain about constant in the controls up to 21 weeks of dust exposure. However, spleen weights of silica-exposed mice were significantly heavier at 12 ($p < 0.05$), 21 ($p < 0.005$), and 39 ($p < 0.05$) weeks than the control mice. Data was expressed (Figure 5) as spleen/body weight ratios. Spleen/body weight ratios of silica-exposed animals differed significantly from the controls at 15, 21 ($p < 0.01$) and 27 ($p < 0.05$) weeks.

Localization of Silica Dust in Tissue

Groups of six mice were exposed to silica dust (at a mean concentration of $4932.25 \pm 235.4\ \mu g/m^3$) for 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39 weeks. After the exposure period, mice were sacrificed and tissues were prepared for observation under light polarizing microscopy and energy dispersive x-ray analysis using scanning electron microscopy.

Light microscopy is the oldest technique used for particle identification (23). Light microscopy has the advantage of being able to distinguish various anatomical sites by staining techniques which cannot be done with SEM. However, the resolution of light microscopy is far inferior to that of SEM. Silica particle identification is not

Table 3. Body and Spleen Weights of Mice After Silica Exposure.

Exposure to Silica Dust (weeks)	Body Weights[a]		Spleen Weights[a]	
	Silica	Control	Silica	Control
3	20.22 ± .84	21.75 ± .91	.106 ± .010	.111 ± .008
9	21.00 ± .71	20.50 ± .51	.120 ± .006	.110 ± .007
15	23.32 ± .38	23.13 ± .61	.126 ± .004[b]	.110 ± .004
21	23.80 ± .45	23.60 ± .79	.129 ± .004[c]	.109 ± .004
27	24.17 ± .39	25.57 ± .49[b]	.114 ± .005	.103 ± .004
33	25.25 ± .57	25.60 ± .70	.122 ± .006	.128 ± .007
39	25.03 ± .69	23.83 ± .51	.170 ± .006[b]	.145 ± .007

[a]Values are expressed as mean ± S.E.M. for six mice.
[b]Significant $p < 0.05$ when compared to control group.
[c]Significant $p < 0.005$ when compared to control group.

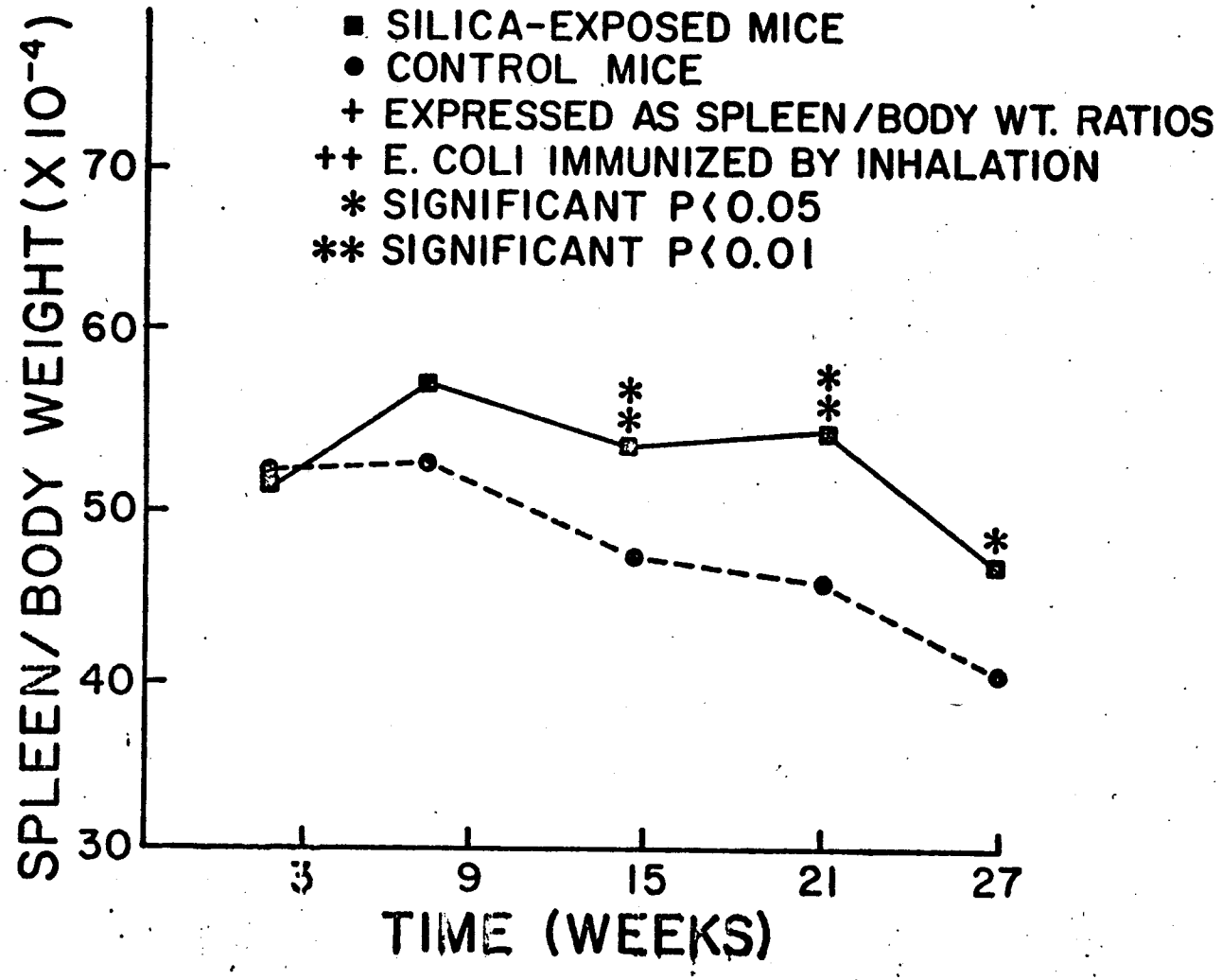

Figure 5. Effects of silica exposure on mouse[+] spleen weights.[++]

possible under ordinary light microscopy. However, silica's birefringent properties allow these particles to be identified with the use of a polarizing lens and allows the particles to be visualized in various areas of pulmonary tissue.

Using polarized light, it was possible to identify silica in isolated macrophages in lung tissue after silica dust exposure. Particles were detected in macrophages at all time periods from 3 to 39 weeks. These dust-filled macrophages usually were located in pleural areas. Silica particles also were found in lymph nodes throughout the same time period of 3 to 39 weeks.

Scanning electron microscopy also can be used to localize silica dust in tissues. In biological tissues such as the lung, much of the lung structure disguises particles and makes identification of the particles difficult. If the mode of backscattered electron (BSE) imaging is used, the higher atomic number of silica over that of biological tissue allows the visualization of silica particles in tissue. In the BSE image the higher atomic number particles, such as silica, are easily recognized. Once the particles are revealed they can be analyzed by x-ray spectroscopy, such as energy dispersive x-ray analysis (EDXA).

Localization of Silica Particles by EDXA

EDXA is an analytical technique which permits simultaneous multielemental analysis of elements, while the tissue is being observed in the scanning electron microscope. This technique was used to localize silica particles in pulmonary and splenic tissue of

silica-exposed animals. EDXA was used to identify sites of deposition of silica particles too small to be detected under ordinary light microscopy. In the lung, however, it was found that before 15 weeks of exposure there were only 1 or 2 particles of silica identified per tissue examined. At 15 weeks, greater numbers of silica particles were detected, up to 0.02% of the volume of the total lung area observed (Table 4). Using polariscopic light microscopy, numerous particles were observed in foamy macrophages at 15 weeks. Using light microscopy small amounts of silica dust also were found previous to 15 weeks. The volume percent of silica particles counted with EDXA over a 27 week period was found to increase gradually to 0.06% (Table 4).

Using polarized light microscopy, aggregations of silica-filled macrophages were observed at 24, 33, 36, and 39 weeks. Mature silicotic nodules that polarized with silica particles, were detected at 39 weeks. The number of silica particles counted under EDXA at 24, 33, 36, and 39 weeks tended to be higher than the groups which had no observable silicotic lesions. However, the volume percent of silica particles counted in the total area examined was not higher than in the groups with no observable silicotic lesions. No silica particles were observed in the controls.

Spleens also were examined for levels of silica in the tissue. After exposure to silica dust for 3, 12, 21, 30, and 33 weeks, variable numbers of silica particles were detected. Large counts of 68 and 83 were found at 3 and 30 weeks, respectively, while insignificant counts (3 and 5) were found at 21 and 33 weeks. Thus, no

Table 4. Energy Dispersive X-ray Analysis of Silica-Exposed Tissue.

Exposure to Silica Dust (weeks)	Silicotic Lesions	Numbers of Silica Particles Counted	Volume % of Silica Particles Counted
		LUNG	
15	-	31	0.02
18	-	28	0.03
24	a	87	0.03
27	-	49	0.06
33	a	83	0.02
36	a	168	0.01
39	a, b	110	0.04
		SPLEEN	
3	-	68	0.03
12	-	20	0.01
21	-	3	0.00
30	-	83	0.04
33	-	5	0.00

[a]Presence of aggregations of silica-filled macrophages under light microscopy.
[b]Presence of silicotic nodules under light microscopy.

relationship between the number of particles found and amount of exposure was found. However, it is possible that an aggregation of silica particles was counted by the x-ray analyzer as one particle. This might explain the extremely low counts found at 21 and 33 weeks after silica dust exposure. No silica particles were detected in the spleens from control mice.

Histopathology of Lung Tissue After Silica Dust Exposure

Groups of six mice were exposed to silica dust (at a mean concentration of 4932.25 ± 235.4 $\mu g/m^3$) for 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39 weeks. After each exposure period mice were sacrificed and tissues were prepared for observation using light and scanning electron microscopy.

Using light microscopy, controls that were sacrificed in the first exposure intervals exhibited little lymphoid infiltration and could be described as "relatively" clean. With increasing time intervals, however, moderate to heavy lymphoid infiltration was seen. From 12 weeks on all controls exhibited some degree of lymphoid infiltration characteristic of an inflammatory reaction (Table 5). The inflammatory reaction seen was characterized by the presence of numerous plasma cells, lymphocytes and polymorphonuclear leukocytes. Often low grade inflammation involving large areas of lung was found. Perivascular and peribronchiolar lymphocyte cuffing was seen in most exposure groups. It is possible that these mice contracted chronic respiratory disease (CRD) of Mycoplasmal etiology which is common in rodent populations (152).

Table 5. Results of Histological Examination of Silica-Exposed and Control Lung Tissue in Mice: Degree of Nonsilicotic Lesion Development.[a]

Weeks of Exposure	Peribronchiolar Cuffing		Perivascular Cuffing		Lymphoid Infiltration	
	Silica	Control	Silica	Control	Silica	Control
3	1.3	0	1.0	1.0	1.7	1.3
6	1.7	0.7	0	0.3	1.0	0.7
9	2.7	0.7	1.7	0.7	2.3	2.0
12	1.7	0.3	1.7	0.7	2.0	3.3
15	3.3	1.3	2.3	0	3.0	2.3
18	2.0	2.0	0.7	3.0	2.7	3.3
21	2.0	2.7	3.0	1.7	2.3	2.7
24	1.7	1.0	1.3	1.7	2.3	2.3
27	2.7	2.0	1.7	2.7	3.0	3.7
30	2.3	0.7	1.3	0.7	2.0	2.0
33	2.0	0.7	1.7	0.7	2.3	2.0
36	1.7	3.0	1.0	2.0	2.0	3.3
39	2.7	0.7	2.0	1.0	2.7	2.3
Total	27.8	15.8	19.4	16.2	28.3	31.2

[a]Degree of lesion severity was determined on a scale of 1 to 5. Average was taken of three animals for each group.

Groups exposed to the silica dust aerosols at a concentration of 4932 $\mu g/m^3$ were found to exhibit an overall increase in cellularity in the first few exposure intervals (3, 6, 9, and 12 weeks). The increase in cellularity was distinctly recognized in peribronchiolar areas (Table 5). The cellularity observed in these animals could be termed a peribronchitis, or peribronchial penumonia, since the inflammation was present in the vicinity of the bronchi and bronchioles. These areas, when observed by light microscopy, contained clusters of macrophages and neutrophils, with macrophages sometimes appearing vacuolated. Single small mononuclear cells resembling lymphocytes also were seen in numerous areas associated with these macrophage clusters.

The first few exposure intervals (3, 6, 9, and 12 weeks) also were characterized by the presence of macrophages in the alveolar septae or adjacent to an alveolar epithelial cell. There were macrophages in all animals, but the extent and the location varied. Occasionally a macrophage was present in the alveolar lumen.

In the mouse there is a respiratory bronchiole interposed between the alvcolar duct and the terminal bronchiole. The terminal bronchiolar epithelium is composed primarily of ciliated cells, nonciliated epithelial cells (Clara cells) and brush cells. The Clara cells are found to be randomly distributed among the more frequent ciliated cells. The third type of epithelial cell, the "brush" cell is very rarely observed. The Clara cell has a centrally located cytoplasmic projection (Figure 6).

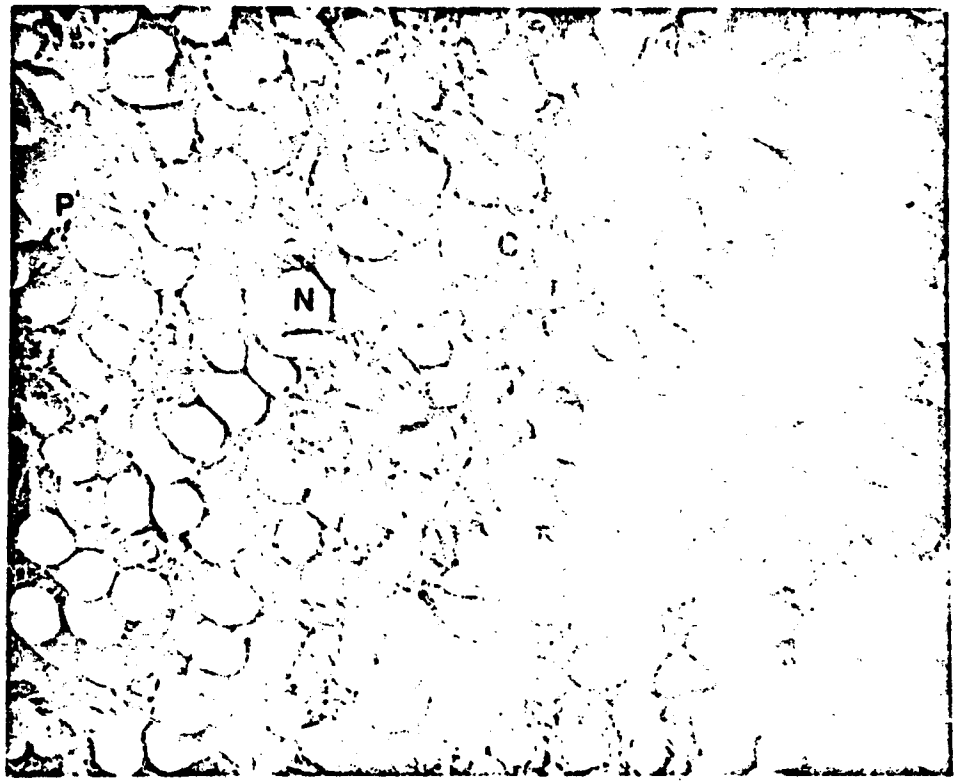

Figure 6. Scanning electron micrograph of bronchiolar epithelium in a nonexposed (control) mouse consisting of nonciliated (N) or Clara cells with a centrally located cytoplasmic projection (P) and ciliated cells (C). x 1000.

When examined by scanning microscopy, the changes seen in the terminal bronchioles of the silica-exposed lung were not extensive up to 15 weeks of exposure. In a minority of animals exposed up to 15 weeks to silica dust, occasional small foci or lymphoid infiltration occurred in alveolar regions. This frequently was observed when there was also peribronchiolar and perivascular cellularity. The absence of even early silicotic lesions by 15 weeks made it necessary to establish whether or not any particulate silica actually had accumulated. For this purpose polarized light microscopy was employed. At 3 weeks, using polarized light, particles could be located having minute birefractive radiance. This form of radiance is known to be produced by silica particles (23). Thus, by 3 weeks, even though no early silicotic lesions were found, silica crystals could be identified in macrophages located in the interalveolar septa or attached to alveolar epithelium which projected into the alveolar lumen, but not in the macrophages located perivascularly or peribronchiolarly. It has been postulated that the presence of macrophages in peribronchiolar and perivascular positions might be in response to the presence of silica particles (47). However, no silica particles were detected in these lesions. The isolated or single macrophages which showed polarizing silica particles were encountered often in the alveolar interstitium adjacent to the pleura.

The scanning electron microscope has great depth of focus and is a good tool for the examination of surface changes. Surface changes observed using the SEM were subtle during the first 15 weeks

of exposure. Clusters and/or single infiltrating cells having a roughened appearance (Figure 7) were present within alveoli. These cells were predominantly macrophages with numerous cytoplasmic projections. A few small mononuclear cells which resembled lymphocytes were observed frequently in areas close to the macrophages.

Overall, up to 15 weeks of silica dust exposure at the concentration of 4932.25 $\mu g/m^3$ did not cause any dramatic changes in the alveolar regions. Sometimes inflammatory cell infiltrations were seen in this area (Figure 7). However, the interalveolar septae and the alveoli did not differ significantly in the exposed animals than in the controls (Figure 8). The more frequent occurrence of macrophages in the silica-exposed animals was noted, but a consistent difference between those and the controls could not be determined.

Thus, up to 15 weeks, in observations using both a light microscope and SEM, the effects of silica dust were subtle. Tissue changes such as alveolar wall thickening and infiltration of inflammatory cells could not be directly attributed to a host's response to the particles, although silica was present in the tissue.

However, by 24 weeks of silica exposure significant tissue changes were present in experimental animals using light microscopy. At this time the first evidence of an early silicotic lesion was observed (Figure 9). As mentioned previously, silica particles were detected in macrophages and in alveolar walls before 15 weeks, however, no accumulations of macrophages were seen. At 24 weeks macrophages were associated with the silica dust and their cytoplasms

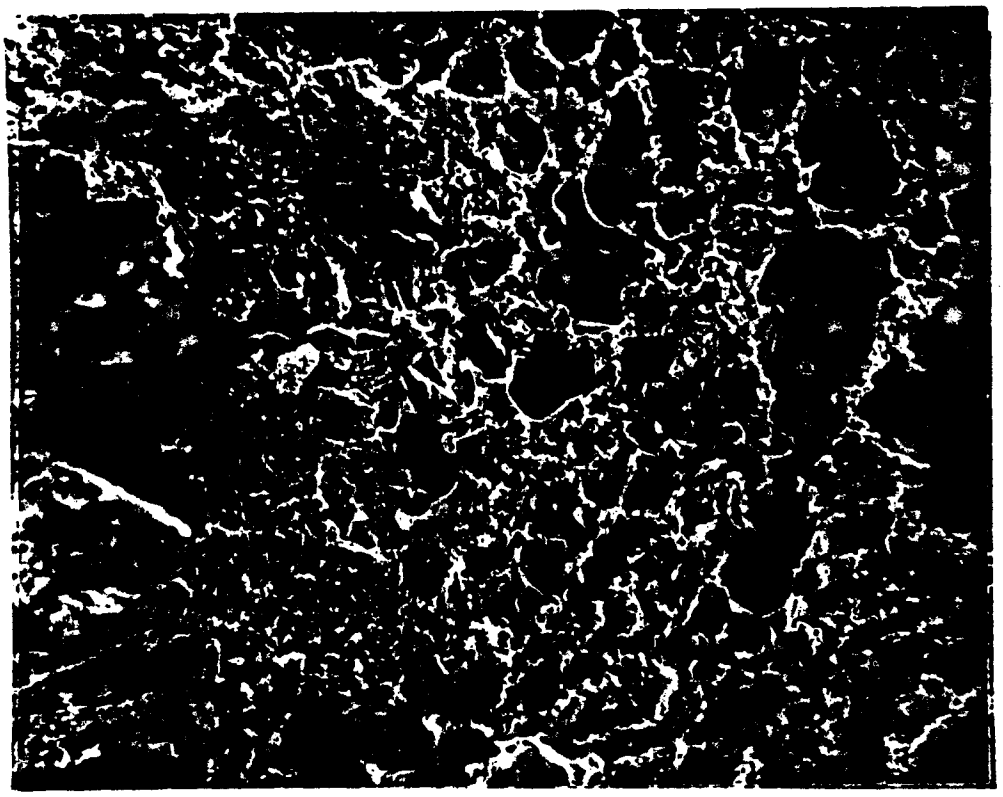

Figure 7. Scanning electron micrograph of the interalveolar septa (IS) and the alveoli (A) of a 15 week silica-exposed mouse lung inhabited by clusters and/or single macrophages (M) in a peribronchial (PB) area. x 200.

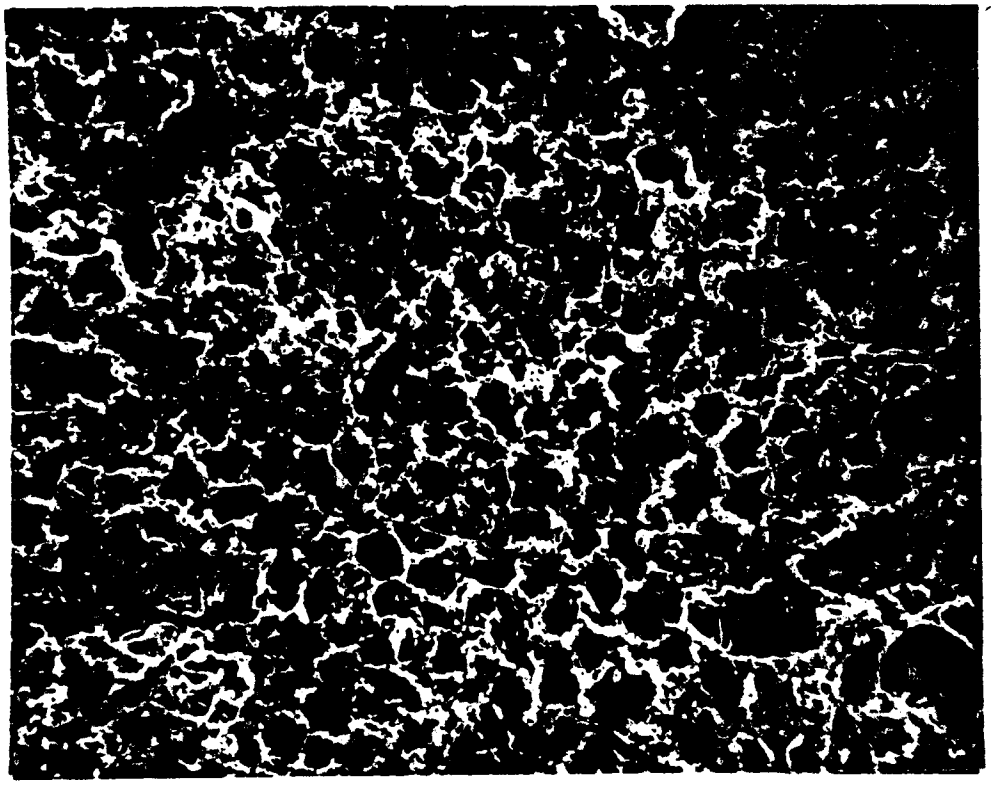

Figure 8. Scanning electron micrograph of the interalveolar septa (IS) and the alveoli (A) of a control mouse lung, sacrificed at 15 weeks. Note the lack of accumulations of macrophages and lymphocytes in this control group as compared to Figure 7. x 200.

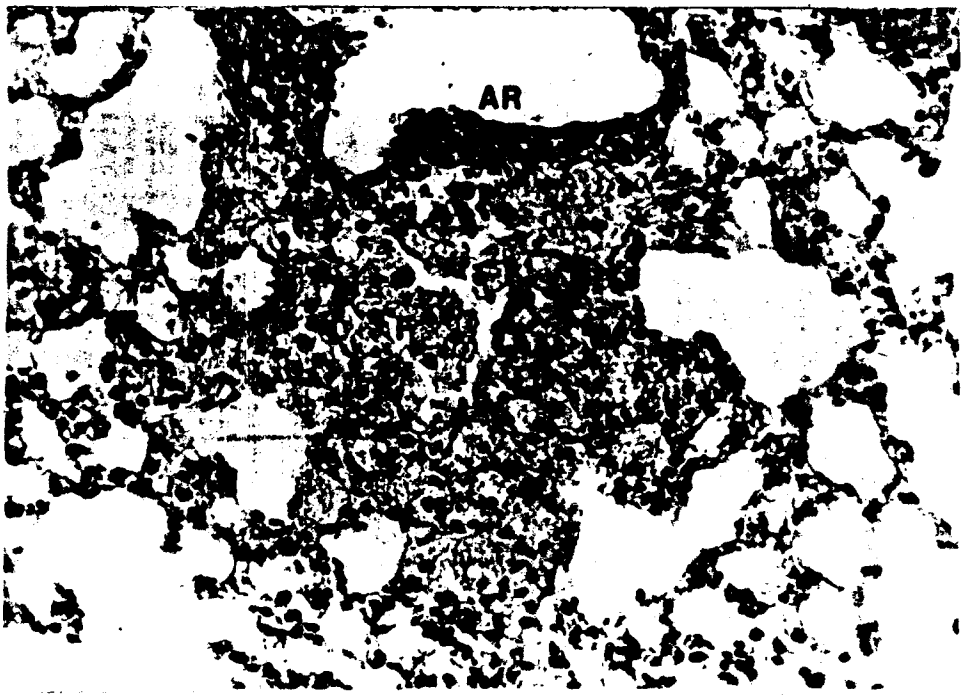

Figure 9. Light micrograph of 24 week exposed lung tissue demonstrating the first evidence of an early silicotic lesion. Silica-filled "foamy" macrophages (F) were found adjacent to an arteriole (AR). H & E Stain. x 400.

were distended and packed with the dust. These cells had a "foamy" appearance and accumulated adjacent to arterioles, bronchioles and bronchi. No foamy cells were located in any other area of the tissue examined. At 24 weeks congestion and thickening of alveolar walls was seen, and in these areas an occasional macrophage was found which contained a small number of particles. In some areas perivascular and peribronchiolar lymphatic cuffing was also evident, but as in earlier groups, particles were not detected in these cells. Hyperplasia of respiratory epithelium in the bronchioles and bronchi was seen, but could not be directly related to the presence of dust.

In subsequent weeks the same foamy cell lesions were found. After 33 weeks of exposure to silica dust there was an accumulation of macrophages and lymphocytes, some of the former being necrotic. One of the foamy cell areas was adjacent to an arteriole which also was "cuffed" by macrophages, lymphocytes and some fibroblasts. After 36 and 39 weeks there also were areas of dust-filled foamy cells. These foamy cells, as in the 33 week group, were located adjacent to major bronchioles (Figure 10).

Later silica exposure groups showed more dramatic changes when examined with SEM. These changes were recognized primarily in the nonciliated bronchiolar epithelial cells or Clara cells. Changes in the ciliated cells were not apparent. The Clara cells, in mice exposed to silica for longer periods were not similar to the Clara cells seen in control animals. In the exposed mice, Clara cells were consistently hyperplastic and bulged into the bronchiolar lumen (Figure 11). Clusters of macrophages were seen, some appearing to

Figure 10. Polarized light micrograph of a 33 week silica-exposed lung demonstrating aggregations of silica-filled "foamy" macrophages (F) in intra- (IB) and peribronchiolar (PB) positions. x 250.

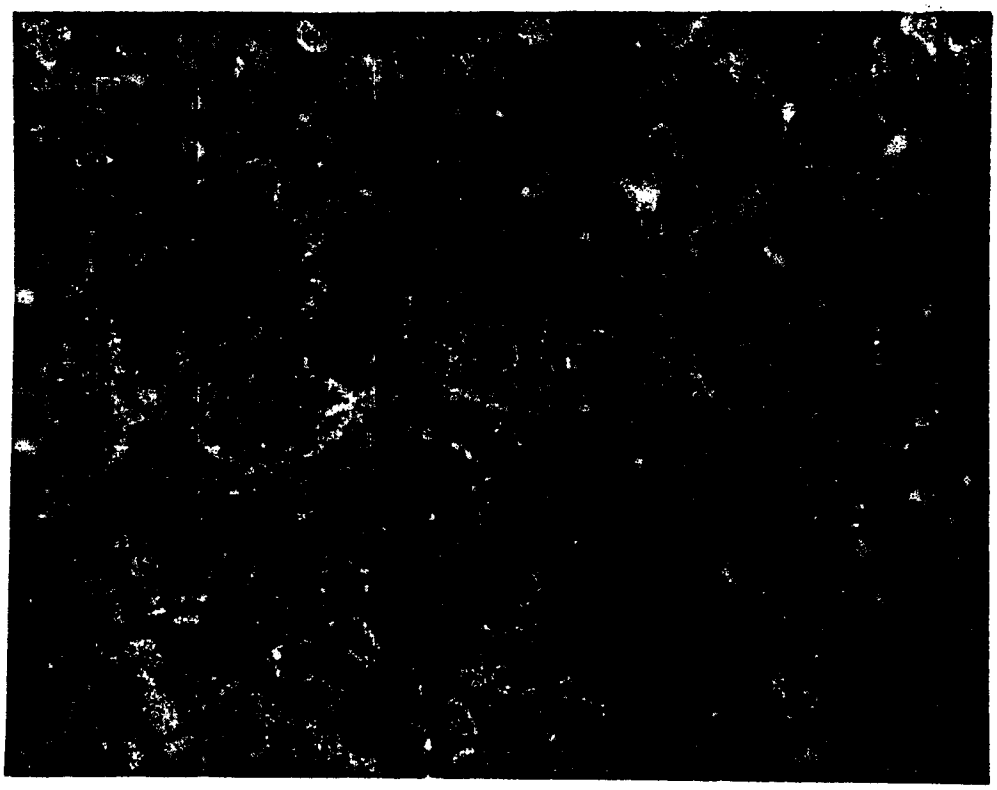

Figure 11. Scanning electron micrograph of bronchiolar epithelium of a 36 week silica-exposed mouse showing hyperplastic (H) and irregularly shaped (I) nonepithelial (Clara) cells. Also seen are the ciliated cells (C) of the bronchiolar epithelium and macrophages (M). x 800.

be "laced" together (Figures 12 and 13). The control lungs at this time period were not void of macrophages but their numbers were very low compared to the number seen in silica-exposed lungs. Thus, pulmonary changes after 24 to 36 weeks were very apparent both using scanning and light microscopy.

After 39 weeks the first evidence of "classical," fibrotic silicotic nodules was seen (Figure 14). Nodules filled with macrophages, lymphocytes and fibroblasts were detected. Accumulations of dust cells were found adjacent to these nodules. Other areas of foamy cell macrophages also were located. These cells also contained silica particles (Figure 15). Unlike the aggregations of foamy cells seen previously, these clusters were located at the periphery of the lung near the pleura. The silicotic nodules that were detected also were found to be at the pleural surface.

Using scanning electron microscopy, alveoli were found to be congested with dust-filled macrophages and lymphocytes. Occasionally macrophages and lymphocytes were seen in areas where alveoli were relatively normal. Peribronchiolar areas were highly congested with plasma cells and lymphocytes (Figure 16). Such a degree of dense cellularity was not encountered in earlier exposure groups. When aggregations surrounding bronchioles were noted in early groups, they were much less dense. Unfortunately, it was not possible to examine the tissue with a light microscope since all of it was prepared for electron microscopy. However, this dense cellularity might suggest the presence of a silicotic nodule.

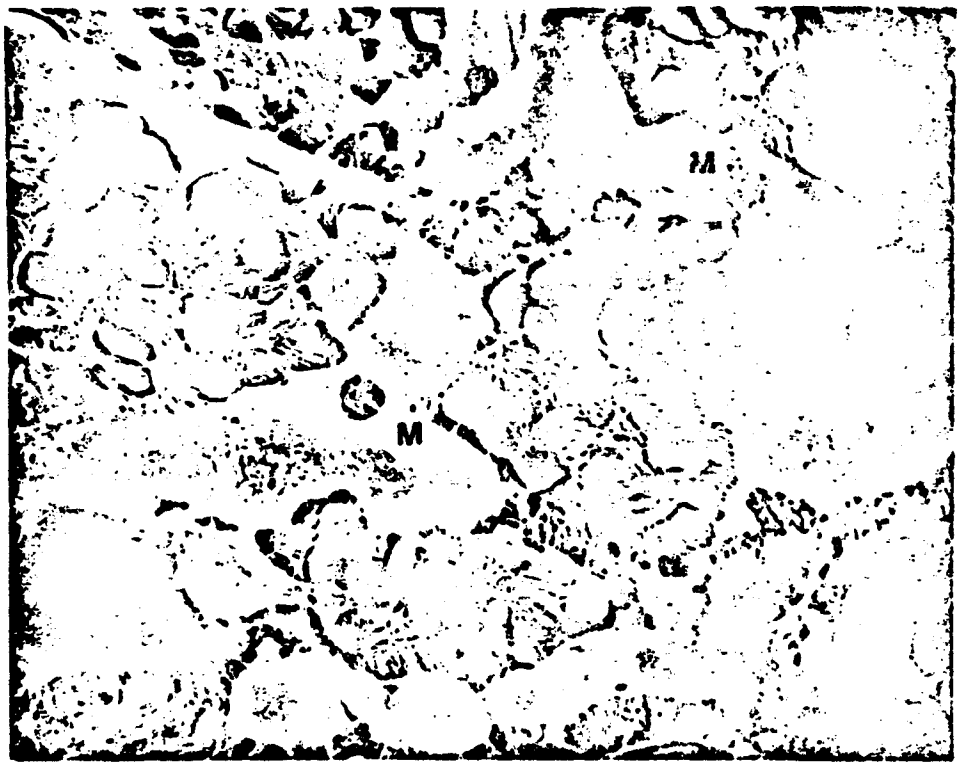

Figure 12. Scanning electron micrograph of bronchial epithelium of a 33 week silica-exposed mouse lung showing macrophages (M) which appear to be "laced" together. x 1000.

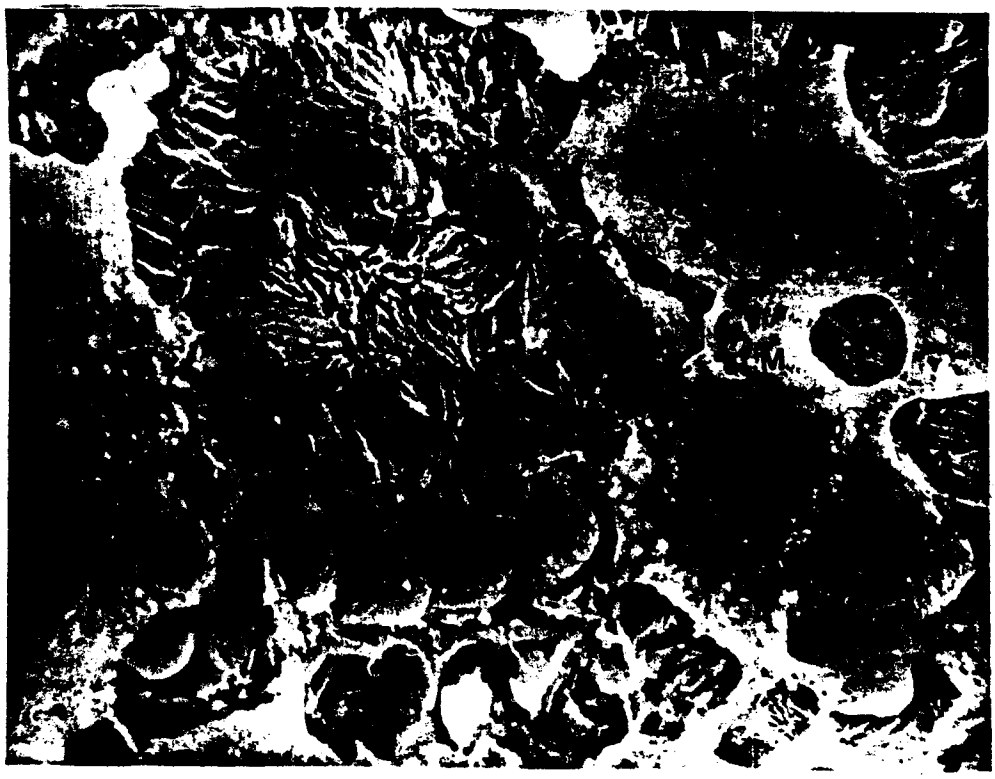

Figure 13. A higher magnification of Figure 12 showing a closer view of the macrophages (M). x 2000.

Figure 14. Light micrograph of a 39 week silica-exposed mouse demonstrating a mature fibrotic silicotic nodule. H & E stain. x 100.

Figure 15. Polarized light micrograph of a 39 week silica-exposed animal showing accumulations of silica-filled dust cells (D) adjacent to a developed silicotic nodule (N). Dust cells and nodules were found concentrated at the pleural surface (PS). x 250.

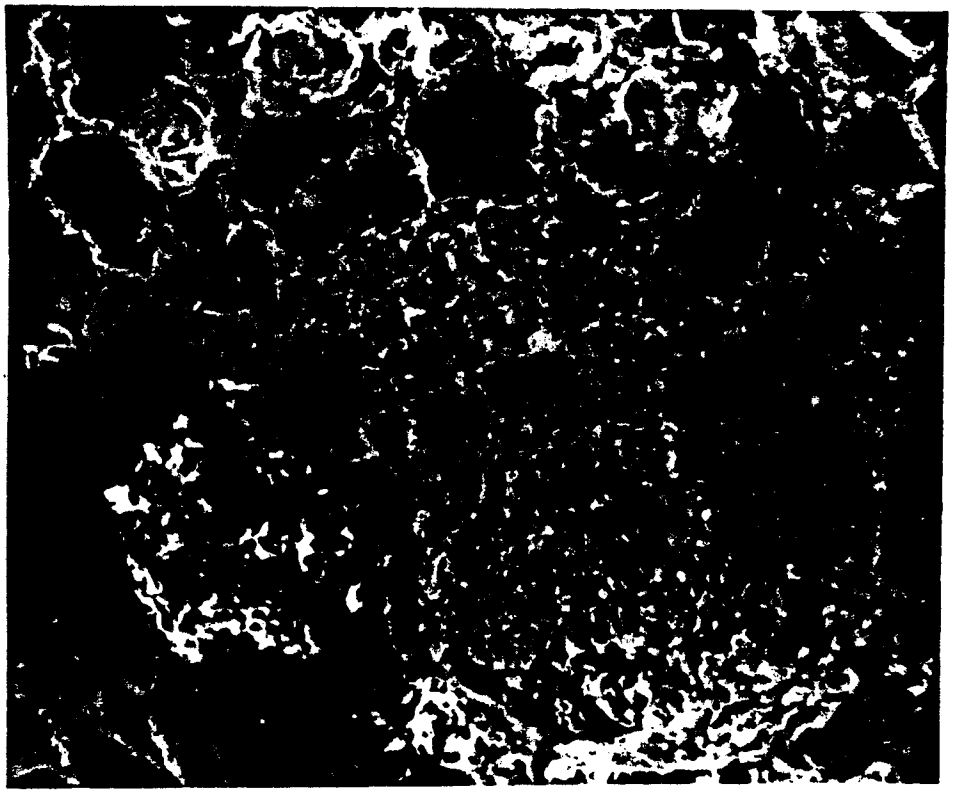

Figure 16. Scanning electron micrograph of a 39 week silica-exposed mouse showing severe peribronchiolar congestion. The cells "cuffing" the bronchiole (B) appear to be plasma cells and lymphocytes. x 500.

The presence of a certain degree of fibrosis with collagen formation is prerequisite before the classical silicotic lesions develops. For this reason, besides a standard hematoxylin and eosin (H & E) stain for light microscopy, Masson's trichrome staining technique for collagen also was used. Using this stain it was determined that these nodules were fibrotic and after 39 weeks at a dosage of 4932 $\mu g/m^3$ silica aerosol, the classical silicotic nodules were present (Figure 17). Not only were these nodules detected in the lung pleural area, but also in the regional mediastinal lymph nodes. Fibrotic nodules in these lymphoid tissues were more advanced than those seen in the lung (Figure 18).

Histopathology of Silica- and Antigen-Exposed Lung Tissue

Animals that were exposed for 3, 9, 15, 21, 27, 33 and 39 weeks to silica and followed by stimulations by antigen aerosols were used for histological investigations. The lesions in these animals were different from those found in lungs exposed to silica only. Much perivascular and peribronchiolar cuffing was evident. Using polarized light, silica dust was detected and great cellular accumulations of macrophages and lymphocytes were present. Areas adjacent to major bronchi were highly congested. After antigen administration the silica-exposed lung tissue showed also the reaction to *E. coli* antigen. In this last group at 39 weeks, there was severe infiltration of the tissue with inflammatory cells (Figure 19).

Figure 17. Light micrograph of a 39 week silica-exposed mouse demonstrating collagen formation in the silicotic nodule. Masson's trichrome stain. x 450.

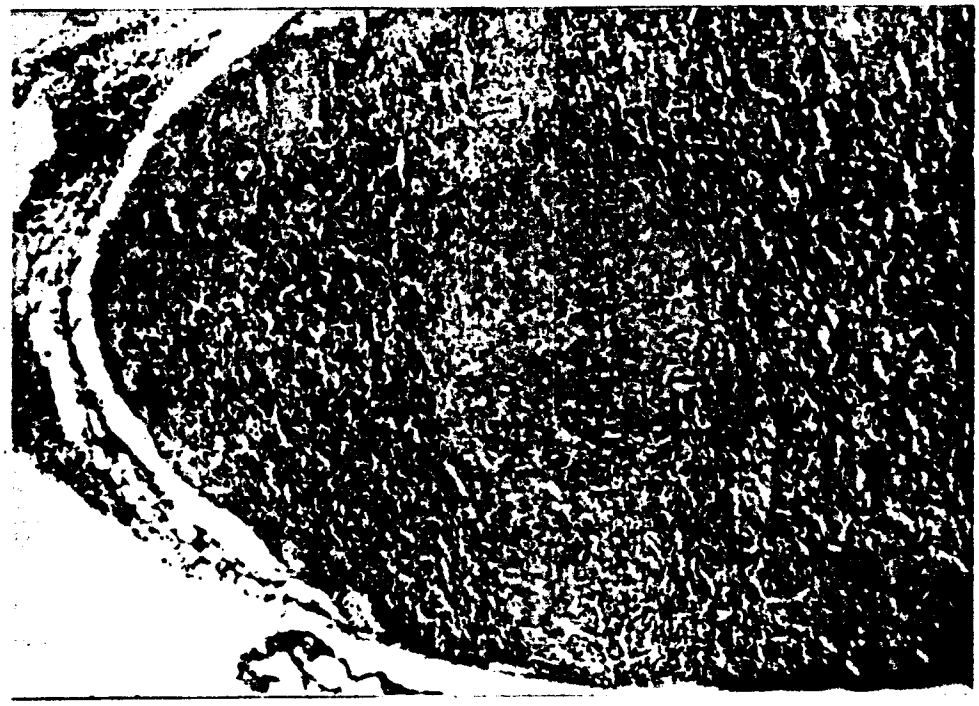

Figure 18. Light micrograph of a 39 week silica-exposed mouse demonstrating fibrotic nodules in the mediastinal lymph nodes. Masson's trichrome stain. x 100.

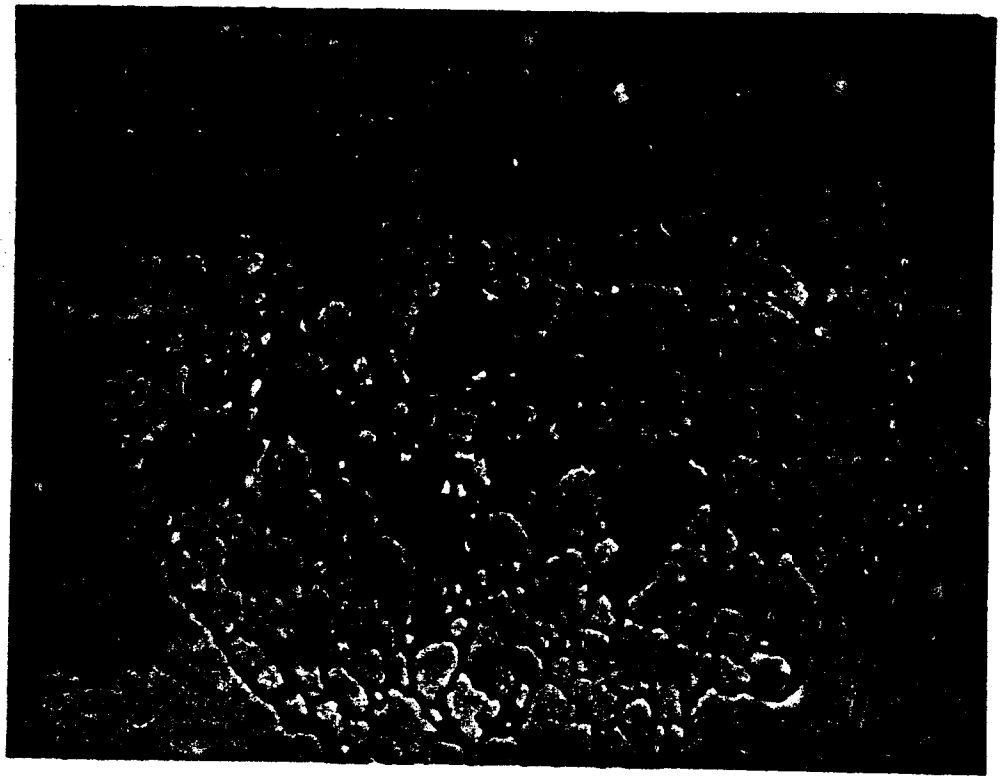

Figure 19. Light micrograph of a 39 week silica-exposed mouse after antigen exposure demonstrating areas adjacent to major bronchi highly congested with inflammatory cells. x 40.

DISCUSSION AND CONCLUSION

Effects of Particle Inhalation

The lung, an organ that is in constant contact with air and has an extremely large alveolar surface area, is very susceptible to the effects of any potentially toxic substances present in the air. Prolonged inhalation of a number of toxic particulates have been shown experimentally to induce toxic effects and epidemiological studies have shown a correlation between the presence of certain particulates in the air and increased morbidity and mortality in people and other animals. A number of diseases and conditions are associated with toxic particulate inhalation and they include: pneumoconiosis, silicosis, some cancers, bronchitis, emphysema, asthma and pneumonias (7, 42, 137).

The effects of toxic particulate inhalation vary according to the pollutant and the intensity of exposure. Damage caused by toxic particulate inhalation can be classified as (1) tissue damage, and (2) cellular changes.

Tissue damage varies from progressive massive fibrosis in anthracosis, to congestive pneumonitis in acute berylliosis. In asbestosis, tissue damage results in the formation of granulomas undergoing fibrosis. Parenchymal scarring and emphysema also are produced. Tissue damage from beryllium dust results in interstitial fibrosing chronic pneumonitis. In chronic berylliosis, granulomas are observed with necrotic centers.

Cellular changes vary according to the toxic particulate inhaled. Cellular changes may be: histological changes, population

changes, and/or immunological changes. These changes are more subtle than tissue changes. Histologically, in asbestosis and chronic berylliosis there is a histiocytic and giant cell reaction leading to the formation of granulomas. In acute berylliosis, there is neutrophilic infiltration which is gradually replaced by macrophages and lymphocytes. In anthracosis, there are aggregations of dust-laden macrophages about the terminal and respiratory bronchioles.

Cellular changes include changes in cell populations. Exposure to a variety of inhaled toxic particulates produces marked changes in the number and activity of leukocytes in the lung. It was found that the immediate effects of exposure to tobacco smoke and lead are to decrease the viability of the pulmonary alveolar macrophage population (69, 70, 71, 116, 151). However, longer exposures usually resulted in an increased population of alveolar macrophages (70, 89). Longer exposures in some experimental animals also caused increased numbers of polymorphonuclear leukocytes. Both short term and long term exposures of toxic particulates were found to decrease pulmonary bactericidal capacity (126 - 131).

Changes affecting humoral and cellular components of the immune response have been evaluated for different contaminants. Acute prolonged exposure to carbon dust results in a severe depression in both local and systemic antibody responses (158). Following moderate periods of carbon dust exposure, systemic cellular immune reactivity exhibited transient stimulation. Systemic effects of carbon dust on B lymphocyte responses however have not been found (157).

Effects of Particulate Silica

The particulate silica, when inhaled, produces tissue damage and cellular changes. Severe pathological changes after silica dust exposure result in the condition of silicosis. The first reaction of the host to silica particles is the accumulation of dust-laden alveolar macrophages within alveolar spaces. Phagocytosis of silica leads to the death of macrophages. A fibroblastic reaction occurs at this location. The accumulation of multinucleated giant cells converts this fibroblastic focus into a granuloma. The classical pathological tissue damage in silicosis is the formation of discrete nodules termed granulomas. The granuloma becomes progressively more fibrotic and is eventually converted into an acellular nodule of concentric layers of hyaline-appearing connective tissue.

The toxic effects of particulate silica on cells are well established (2-5, 19, 20, 76, 97). Silica particles have been shown in numerous investigations to be specific macrophage toxins. Cultures of alveolar and peritoneal macrophages have reproduced the *in vivo* cytotoxic effects of silica. Macrophage ingestion of silica has been found to increase phagosomal membrane permeability. Subsequently, there is leakage of hydrolytic enzymes into the cytoplasm and the macrophages are rapidly killed and lysed. Silica particles liberated from the cells are then able to lead to repeated cycles of macrophage killing and lysing (9, 143). The mechanisms accounting for increased permeability has been found to be the hydrogen bonding of silicic acid with lipid and protein components of the phagosomal membranes (34, 111, 112, 148).

Silicic acid liberated into the tissues from silica has been postulated to bring about collagen deposition. Deposition of collagen within granulomatous foci eventually leads to the classical pathological picture of silicosis.

Effects of Silica on the Functions of the Immune System Responses

In addition to the cytotoxic effects of silica particles on the macrophages, silica has been reported to have an in vivo effect on cell-mediated allograft rejection (117, 118). Silica has been reported to abrogate hybrid and allogeneic resistance to bone marrow grafts (91). Studies of the direct effects of silica particulates on immune responses however have not been extensive. Miller and Zarkower (102-104) and this study have extended the studies dealing with the effects of experimentally produced silicosis on several murine immunological parameters. The possible effects of experimental silicosis on the immunocompetence of the thymus-derived (T cell) and bone marrow-derived (B cell) lymphocyte populations was examined. In addition, the effect of silica as related to the mechanism of antigen processing was investigated.

When mice were exposed to silica dust for 3, 7, 12, and 21 days, a significant decrease of antibody-forming cells was present in the spleen, when a T cell independent antigen, E. coli 055:B5 LPS was used. Therefore, the results pointed to a significant depression of B cell response. Longer periods of exposure to silica dust resulted in progressive decreases in B cell response in both spleens and

mediastinal lymph nodes. Removal of mice from silica exposure chambers did not reverse the effects readily and a highly significant decrease in the response to LPS was found three months later (100, 101).

The suppression of the immune response to E. coli aerosols was postulated to be (1) an interference with antigen introduction (in the silica-stressed lung) or, (2) interference with antigen processing (because of the silica-damaged macrophages) or lastly, (3) a decreased ability of the B cell population to respond to antigenic stimulation. When the silica-stressed lung was bypassed with either intraperitoneal or intravenous injection of E. coli antigen, the decrease in response was not as great as that seen consistently after aerosol stimulation (104). The greater suppression seen after aerosol immunization indicated the more severe changes that silica may cause in lung tissue.

It was the attempt of this investigation to correlate the presence of silica with functional changes. There are a variety of factors that could have been involved in causing the effects seen. Possible factors inducing functional changes could include: (1) the presence of silica particles in the affected tissues; (2) solubility of silica with silicic acid being dispersed throughout lung tissue and other organs, and; (3) cytoxicity of silica on macrophages. This last factor, the effect of silica on macrophages, could result in (a) paralysis of macrophage function; (b) mobilization of macrophages into lung and mediastinal lymph nodes and very efficient trapping of

antigen, in turn preventing the antigen from reaching other lymphoid organs; and (c) lysis of macrophages with the release of substances that may influence the functioning of other cells. With these mechanisms in mind an attempt was made to correlate the localization of silica deposition and the histopathological changes, with the concurrent functional changes in immune responsiveness.

When Miller and Zarkower (102) measured the number of PFC/spleen after the aerosol administration of E. coli LPS antigen, an immunosuppressive effect of inhaled silica dust on the response of B lymphocytes of the spleen was found. When LPS was administered by an intravenous route, and thus bypassing the silica-stressed lung area, the suppression of the number of PFC/spleen was less but still statistically significant.

In this investigation it was found that longer periods of dust exposure (up to 27 weeks) resulted in progressive decreases in B cell responses in the spleen after aerosol administration of antigen. However, when mice exposed for 39 weeks to silica dust were given E. coli antigen intravenously, the numbers of antibody-forming cells in the spleen were not significantly affected. The immunosuppression of responsiveness to the antigen was possibly the result of silica's cytotoxic effects on macrophage functions of phagocytosis and antigen processing, and/or, silica's effect on the dissemination of antigen from the silica-stressed lung to other lymphoid tissues.

Effects of Silica Deposition and Histopathological Changes

In an attempt to understand the factors involved in

silica-induced changes, it was necessary to correlate immune-functional changes with silica deposition and histopathological changes in lung tissue. Examination of lung tissue at various time intervals after aerosol dust exposure for the presence of silica dust, and evaluation of the course of silicotic lesions in the murine model was done. This investigation was designed to establish a chronic type silica pathology, which develops in industrial conditions after a moderate exposure to the dust extending for a period of 20 to 40 years.

This investigation confirmed previous work in other animal systems that emphasized the pulmonary damage caused by silica dust (35, 79, 149). In this study, however, a much lower dosage and a different model, the mouse, was employed. Other workers have produced the typical compact nodules in rats after exposure in dust chambers for periods as long as one year (57). Ziskind et. al. (159) reported that nodules containing significant amounts of collagen also have been produced in chronically exposed monkeys.

The developing silicotic lesion encompasses the stages of: (1) focal collections of dust cells; (2) concentrations of fibroblasts; and (3) collagenous nodules.

Using polarized light microscopy, silica particles were found in isolated macrophages at three weeks. These particles were not connected with any overt lung pathology. Using energy dispersive x-ray analysis (EDXA), silica particles were detected in the lung from 15 to 39 weeks. In those exposure groups (24, 33, 36, and 39 weeks) showing pronounced silica pathology under light microscopy, more silica

particles were detected using EDXA. Using EDXA, however, no relationship could be drawn between the increase in particle deposition over the time of dust exposure.

Using polarized light microscopy, the first stage of the developing silicotic lesion was observed in the form of dust cells containing large amounts of silica. This occurred after 24 weeks of dust exposure. Focal collections of dust cells have been widely reported in the literature (46, 58, 79, 98, 109). Excessive amounts of retained dust in the alveoli cause destruction of the alveolar epithelium causing an "alveolar ulcer." The alveolar wall interstitium then is exposed with its contained macrophages. These phagocytes engulf the silica and are subsequently detached or remain adherent to the alveolar wall. Recruited macrophages adhere to this site and form an aggregation of foamy cells which protrude into the lumen. The aggregation of such macrophages may become surrounded by a layer of regenerating alveolar epithelial cells. The collection of dust-laden macrophages or foamy cells then occupies an interstitial position in the alveolar wall.

In most cases this dust cell collection has been followed by a concentration of fibroblasts leading to definite collagen nodules. Collections of silica-containing dust cells have been observed in 24, 33, 36, and 39 week exposure groups. Fibroblastic areas in these groups were found adjacent to light-polarizing collections of dust cells. Very small concentrations of fibroblasts also were associated with the peripheral areas of developing collagenous nodules. At 39 weeks,

collagenous nodules were seen, as demonstrated by Masson's trichrome stain. These nodules contained macrophages, lymphocytes and a certain degree of fibrosis.

Relationship of Clearance and Deposition of Silica to Histological And Immunological Changes

In the exposure groups it was important to identify the areas in which the lesions developed in reference to silica deposition to help understand the mechanisms involved. The peribronchiolar areas in the lung tissue were the first developing areas of early silicotic lesions (presence of foamy macrophages). This was in agreement with most of the other descriptions of early lesions (45, 48, 49, 50, 57, 59, 62, 109, 110). However, the final collagenous nodule that was found at 39 weeks, developed in areas of the lung adjacent to the pleura. Throughout all exposure intervals silica particles were found consistently near pleural areas. It has been demonstrated that silica particles can reach the tissue of the distal alveoli either carried by macrophages (28-30) or less commonly as a result of the mechanical movements of the lungs (138). Death of these macrophages or foamy cells usually leads to the gradual formation of nodules adjacent to respiratory bronchioles and arterioles. These nodules also are frequently found lying near the lymphatics surrounding small veins. In our experiments the dust cells were in peribronchiolar and perivascular positions. However, most silica particles and the collagenous silicotic nodules were found proximal to the pleura.

It might be speculated that in the murine model there is a constant movement of silica particles towards the lymphatics of the

pleura. Lauweryns and Baert (87) in a recent review, support the major role of lymphatic clearance in the removal of particulates from the lung. This might serve to explain the formation of collagenous nodules, fibroblasts, and dust cells adjacent to the pleura in later exposures. In earlier exposures, however, such lesions occupied a definite peribronchiolar position.

The median particle size of the silica dust generated in the environmental chambers was 2 μm. At this size alveolar deposition is about 80% of the total particles inhaled (55, 155). Not all of the 80% of the deposited particles remain on the internal surface of the lungs, some enter the alveolar lining and become sequestered in interstitial tissue or enter the lymphatic tissue (138). Although Gross (45) states that lymphatic clearance accounts for only about 10% of cleared dust, other investigators (87) determined that lymphatic clearance is size dependent and dust particles which are sufficiently small penetrate the alveolar membrane and are then carried by tissue fluid toward the lymphatics.

The presence of well-developed fibrotic nodules in the mediastinal lymph nodes also suggests that the lymph nodes account for a major amount of clearance of silica particles. Enlargement of lymph nodes, especially mediastinal nodes, was frequent. Ziskind et. al. (159) stated that this often precedes pulmonary nodulation. Weller and Ulmer (152) found after 22 months of inhalation of silica in rat lungs, changes in the pulmonary lymph nodes were similar to those in the lungs. The granulomas were well organized and developed with a

moderately dense network of reticular fibers and collagenous fibers. Observations in polarized light showed no disintegration of silica particles in these nodes.

The results of this study implicate the major role of lymphatic clearance in murine lungs when the particles are small and administered for a long time. Two of the main postulates of Lauweryns and Baert (87) might be considered likely in this animal model system. They are: (1) silica particles are absorbed in the alveolar lumen by alveolar macrophages which could leave the alveoli via the interstitium to the pulmonary lymphatics; or (2) silica particles alone are removed from the interstitium via the pulmonary lymphatics. The evidence of foamy dust-filled macrophages anatomically adjacent to lymphatic-rich areas would support this first postulate. The cytotoxicity of silica for macrophages (2-5) would support this second postulate of the lymphatic clearance of "free" particles.

However, tracheobraonchial clearance also is of great importance and is established to be the major (90%) clearance mechanism (12-14, 81). Using light microscopy, the paucity of macrophages in alveoli was striking in the first few exposure groups. Using SEM, macrophages also were rarely detected in alveolar duct regions. At the same time periods, macrophages frequently were seen in bronchioles and bronchi.

Ferin (26, 27) proposed that lung clearance depends specifically on the alveolar macrophages coupled to the ciliary-mucous transport process of the tracheobronchial system. It is well established that particles within free alveolar macrophages are removed to the ciliated part of the lung. Although macrophages frequently were not

detected in tracheobronchial areas in early exposure groups using light microscopy, using SEM macrophages were abundant in tracheobronchial areas by 15 weeks. Light microscope investigation might implicate the major role of lymphatic clearance, but SEM also asserts the role of tracheobronchial clearance in our murine system.

By 39 weeks, prominent clustering of macrophages in the bronchial region was observed using the SEM. At this time, macrophages were located in alveolar walls and interstitium, however, they were not as numerous as in the bronchial areas. Alterations in the surface height of the cytoplasmic luminal projections of the nonciliated epithelium also was evident at this 39 week exposure group. Ozone has been found to induce a proliferative response in nonciliated (Clara) cells of mice with the formation of micronodules projecting into the bronchiolar lumen (135, 136). However, the ciliated cells of the bronchioles were not affected throughout the entire silica exposure period. The cilia of the experimental animals were approximately of the same uniform length, diameter and density as the control cilia.

After 39 weeks there was seen, using the SEM, dense accumulations of cells adjacent to the bronchiole. This area could represent a silicotic nodule but the inability to examine it with a light microscope renders the confirmation of this nodule impossible. However, this cellularity does suggest a stimulation of the reticuloendothelial system and a possible involvement of the lymphatics in that area.

Anatomically, two lymphatic drainages serve the lung parenchyma: "deep-set" drainage and pleural drainage (87, 108). The

deep-set drainage consists of the periarterial, perivenous and peribronchiolar lymph vessels. The major drainage of the alveolar region is associated with the periarterial and peribronchiolar lymph vessels. Pulmonary lymphatic anatomy is not well established at present. Tobin (142) found that pulmonary lymphatics accompany branches of the pulmonary arterioles and venules, and are adjacent to alveolar walls. Others (84 - 86) have found lymphatic capillaries between the alveolar walls and the interlobular pleural, peribronchiolar and perivascular connective tissue sheets. Macklin (93, 94) found that "sumps," i.e., "the beginning of lymph vessels" were at the level of the alveolar bronchiole or alveolar duct.

Dust particles have been found to penetrate the epithelial lining of the distal respiratory bronchioles overlying lymphoid foci (lymphoid sumps) and then to enter the lymphatic channels with which these structures were associated (95). Spencer (138) suggested that the majority of dust particles are engulfed by macrophages but a good amount of particles are probably propelled by microcurrents of tissue fluid aided by respiratory movements towards the distal terminations of the lymphatic channels situated in the peribronchiolar, periarterial, septal and pleural tissues. Silica particles ultimately reach the blood stream where these particles may be ingested by reticuloendothelial cells localized in the liver and spleen (41, 138).

Spontaneous pathogenic changes also occurred throughout the 39 week test period. Lymphoid infiltrations were found in about 95% of the animals observed. A cellular infiltration of the alveolar

walls was observed in at least one area of lung tissue from each animal, both control and experimental. Rarely was an animal found which possessed an entirely "clean" lung.

For positive interpretation of dust-exposed lung changes, Weller and Ulmer (152) suggested the use of specific-pathogen free (SPF) animals maintained in isolated and air conditioned units. These SPF animals were found to lack completely the bronchiectasis, peribronchial cell linings and the cellular infiltrations of the alveolar septa that make the interpretation of silicotic lung pathology difficult. However, others (50, 107) have reported that the lung tissue response to silica was similar for both SPF and standard rats. In the standard rats more stimulation of the reticulo-endothelial system by the silica was noted as perivascular cuffing of plasma cells and lymphocytes were observed in regions where dust was not present. However, the clearance rate of silica was not significantly different, and silica produced a typical granulomatous reaction in both groups.

In this study an attempt was made to relate the obvious changes in immune responsiveness due to silica with silica deposition and concurrent histopathological changes in lung tissue and lymph nodes. Immune function was significantly depressed at three weeks. At this three week time period silica could be localized in isolated macrophages. However, typical silica pathology in the form of classical granulomatous lesions was not observed until 39 weeks.

At three weeks, the only visible evidence of a direct effect on lung tissue that might explain the change in immune function was the presence of silica deposited in the lung. It might be speculated that

the initial exposure to silica dust compromised the resident macrophage population. The effect of silica as a macrophage toxin seems to be the most likely mechanism inducing functional changes. It might be that the effects of silica were threefold. First, there was a paralysis of macrophage function. In this way the macrophage-antigen processing system was affected, thus, causing the suppression seen in immune responsiveness. This caused a secondary effect of mobilization of macrophages into the lung and mediastinal lymph nodes. This mobilization in turn, allowed efficient trapping of the antigen and subsequent prevention of the dissemination of the antigen to other lymphoid organs. The continuous immunosuppression or "unresponsiveness" seen in PFC in the spleen and mediastinal lymph nodes over 27 weeks, might be explained by macrophage mobilization in the lung. The third effect, which is the release of substances from lysed macrophages influencing other cells, also is a likely mechanism. Possibly a substance is released which causes changes in B cell immunocompetence.

In conclusion, it must be stated that this is an oversimplification. The role of the lung as a macrophage-lymphoid organ responding to environmental stress from particulates has been investigated only recently. Much more research into the effects, both pathological and immune-functional, of silica's presence in lung tissue and lymphoid cells and organs is needed to elucidate the ramifications of chronic silica dust exposure on the murine model.

BIBLIOGRAPHY

1. Albert, R. E. and M. Lippmann. 1972. Factors influencing dust retention in the pulmonary parenchyma. Ann. N. Y. Acad. Sci. 200, 37.

2. Allison, A. C. 1970. On the role of macrophages in some pathological processes. In Mononuclear Phagocytes, Van Furth, R. (ed.), Blackwell Scientific Publishers, Oxford, pp 422-444.

3. Allison, A. C. 1974. Pathogenic effects of inhaled particles and antigens. Ann. N. Y. Acad. Sci. 200, 299.

4. Allison, A. C., J. S. Harington and M. Birbeck. 1966. An examination of the cytotoxic effects of silica on macrophages. J. Exp. Med. 124, 141.

5. Allison, A. C., J. S. Harington, M. Birbeck and T. Nash. 1965. Observations on the cytotoxic action of silica on macrophages. In Inhaled Particles and Vapours Vol. 2., Davies, C. N. (ed.), Cambridge University Press, Oxford, pp. 121-129.

6. Anderson, T. F. 1951. Techniques for the preservation of three dimensional structure in preparing specimens for the electron microscope. Trans. N. Y. Acad. Sci. 13, 130.

7. Ayres, S. M. and M. E. Buehler. 1970. The effects of urban air pollution on health. In Clinical Pharmacology and Therapeutics, Vol. II, No. 3., C. V. Mosby Co., St. Louis, pp 337-371.

8. Berkley, C., J. Churg and I. J. Selikoff. 1965. The detection and localization of mineral fibers in tissue. Ann. N. Y. Acad. Sci. 132, 48.

9. Bey, E. and J. S. Harington. 1971. Cytotoxic effects of some mineral dusts on syrian hamster peritoneal macrophages. J. Exp. Med. 133, 1149.

10. Brain, J. D. 1970. Free cells in the lungs. Arch. Intern. Med. 126, 477.

11. Brieger, H. and P. Gross. 1966. On the theory of silicosis. I. Coesite. Arch. Environ. Health, 13, 38.

12. Camner, P., K. Philipson and L. Friberg. 1972. Tracheobronchial clearance in twins. Arch. Environ. Health, 24, 82.

13. Camner, P., K. Philipson, B. Holma, B. Larsson and J. Svedberg. 1971. Human tracheobronchial clearance studies. Arch. Environ. Health 22, 444.

14. Camner, P., K. Strandberg and K. Philipson. 1976. Increased mucociliary transport by adrenergic stimulation. Arch. Environ. Health 32, 79.

15. Caplan, A. 1953. Certain unusual radiological appearances in the chest of coal miners suffering from rheumatoid arthritis. Thorax 8, 29.

16. Casarett, L. S. and P. S. Milley. 1964. Alveolar reactivity following inhalation of particles. Health Phys. 10, 1003.

17. Cepellini, R. and B. Pernis. 1958. Presence of plasma globulins in the hyaline tissue in cases of silicosis. Nature 181, 55.

18. Cohen, A., D. P. Marlow and G. E. Garner. 1968. A rapid critical point method using fluorocarbons ("freons") as intermediate and transitional fluids. J. Microsc. 7, 331.

19. Comolli, R. 1967. Cytotoxicity of silica and liberation of lysosomal enzymes. J. Path. Bact. 93, 241.

20. Comolli, R. and A. Perin. 1963. In vitro action of silicogenic and non-silicogenic dusts on macrophage metabolism. Proc. Soc. Exp. Biol. Med. 113, 289.

21. Crider, W. L., N. P. Barkley and A. A. Strong. 1968. Dry powder aerosol dispersing device with long time output stability. Rev. Sci. Instrum. 39, 152.

22. Curran, R. C. and E. V. Roswell. 1958. The application of the diffusion-chamber technique to the study of silicosis. J. Path. Bact. 76, 561.

23. DeNee, P. B. 1976. Identification and analysis of particles in biological tissue using SEM and related techniques. IITRI/SEM pp. 461-468.

24. Dresser, D. A. and H. H. Wortis. 1967. Localized haemolysis in gel. In Handbook of Experimental Immunology, Weir, D. M. (ed.), F. A. Davis Co., Philadelphia, pp. 1,054-1,067.

25. Fallon, J. T. 1937. Specific tissue reaction to phospholipids: A suggested explanation for the similarity of the lesions of silicosis and pulmonary tuberculosis. Canad. Med. Ass. J. 36, 223.

26. Ferin, J. 1967. The alveolar clearance mechanism of retained particles. In Inhaled Particles and Vapours Vol. 2., Davies, C. N. (ed.), Cambridge University Press, Oxford, pp. 181-187.

27. Ferin, J. 1972. Observations concerning alveolar dust clearance. Ann. N. Y. Acad. Sci. 200, 66.

28. Ferin, J., J. R. Coleman, S. Davis and B. Morehouse. 1976. Electron microprobe analysis of particles deposited in lungs. Arch. Environ. Health 32, 113.

29. Ferin, J. and L. J. Leach. 1976. The effect of amosite and chrysotile asbestos on the clearance of TiO_2 particles from the lung. Environ. Res. 12, 250.

30. Ferin, J., G. Urbankova and A. Vickova. 1965. Pulmonary clearance and the function of macrophages. Arch. Environ. Health 10, 790.

31. Field, C., J. L. Allen and H. Friedman. 1970. The immune response of mice to Serratia marcescens LPS or intact bacteria. J. Immunol. 105, 193.

32. Friedman, R. L. and R. J. Moon. 1977. Hepatic clearance of Salmonella typhimurium in silica-treated mice. Inf. and Imm. 16, 1,005.

33. Funahashi, A., K. Pintar and K. A. Siegesmund. 1975. Identification of foreign material in lung by energy-dispersive x-ray analysis. Arch. Environ. Health 30, 285.

34. Gabor, S., Z. Anca and E. Zugravu. 1975. In vitro action of quartz on alveolar macrophage lipid peroxides. Arch. Environ. Health 30, 499.

35. Gardner, L. U. 1932. Studies on experimental pneumokoniosis. VII. Inhalation of quartz dust. J. Ind. Hyg. 14, 18.

36. Gardner, L. U. 1935. Experimental production of silicosis. U. S. Pub. Health Repts. 50, 695.

37. Gil, J. 1971. Ultrastructure of lung fixed under physiologically defined conditions. Arch. Intern. Med. 127, 896.

38. Gil, J. and E. R. Weibel. 1968. The role of buffers in lung fixation with glutaraldehyde and osmium tetroxide. J. Ultrastr. Res. 25, 331.

39. Gil, J. and E. R. Weibel. 1970. Improvements in demonstration of lining layer of lung alveoli by electron microscopy. Resp. Physiol. 8, 13.

40. Gil, J. and E. R. Weibel. 1971. Extracellular lining of bronchioles after perfusion-fixation of rat lungs for electron microscopy. Anat. Rec. 169, 185.

41. Godwin, M. C. and J. Jagatic. 1970. Asbestos and mesotheliomas. Environ. Res. 3, 391.

42. Goldsmith, J. R. 1968. Effects of air pollution on human health. In Air Pollution Vol. 1., Stern, A. C., (ed.), Academic Press, New York. pp. 547-615.

43. Goldstein, E., G. M. Green and C. Seamons. 1969. The effect of silicosis on murine pulmonary resistance to experimental aerosol infection. J. Inf. Dis. 120, 210.

44. Goldstein, P., E. A. J. Svedmyr and H. Wigzell. 1971. Cells mediating specific in vitro cytotoxicity. I. Detection of receptor bearing lymphocytes. J. Exp. Med. 134, 1,385.

45. Gross, P. 1964. The processes involved in the biological aspects of pulmonary deposition, clearance and retention of insoluble aerosols. Health Phys. 10, 995.

46. Gross, P., D. C. Braun and R. T. P. de Treville. 1972. The pulmonary response to coal dust. Ann. N. Y. Acad. Sci. 220, 155.

47. Gross, P. and H. Brieger. 1966. Silicotic bronchiolitis obliterans--a focal clearance failure. Arch. Environ. Health 12, 5.

48. Gross, P., E. A. Pfitzer and T. Hatch. 1966. Alveolar clearance: its relation to lesions of the respiratory bronchiole. Amer. Rev. Resp. Dis. 94, 10.

49. Gross, P. and E. B. Tolker. 1966. Dust particles in lung sections. Arch. Environ. Health 12, 212.

50. Gross, P., M. L. Westrick and J. M. McNerney. 1956. Silicosis: the topographic relationship of mineral deposits to histological structures. Amer. J. Pathol. 32, 739.

51. Gross, P., M. L. Westrick and J. M. McNerney. 1958. Experimental silicosis: the morphogenesis of the silicotic nodule. Arch. Industr. Health 18, 374.

52. Gross, P., M. L. Westrick and J. M. McNerney. 1958. Progression of experimental silicosis in the rat. Amer. Industr. Hyg. Ass. J. 19, 201.

53. Gross, P., M. L. Westrick and J. M. McNerney. 1958. Silicosis: the effect of spontaneous pneumonia upon the distribution of silica in rat lungs. Lab. Invest. 7, 348.

54. Gye, W. E. and W. J. Purdy. 1922. The effects of repeated intravenous injections of colloidal silica on rabbits. Brit. J. Exp. Pathol. 3, 86.

55. Hatch, T. F. 1961. Distribution and deposition of inhaled particles in respiratory tract. Bac. Rev. 25, 237.

56. Hefferman, T. 1963. Further thoughts on silicosis. Tubercle 34, 246.

57. Heppleston, A. G. 1962. The disposal of dust in the lungs of silicotic rats. Amer. J. Pathol. 40, 493.

58. Heppleston, A. G. 1963. The disposal of inhaled particulate matter; a unifying hypothesis. Amer. J. Pathol. 42, 119.

59. Heppleston, A. G. 1967. Atypical reaction to inhaled silica. Nature 213, 119.

60. Heppleston, A. G. 1969. The fibrogenic action of silica. Brit. Med. Bull. 25, 282.

61. Heppleston, A. G. 1970. The pathogenesis of silicosis. In Pneumoconiosis: Proceedings of the International Conference, Johannesberg, 1969. Shapiro, H. A. (ed.), Oxford University Press, London. pp. 496-498.

62. Heppleston, A. G. 1972. The pathological recognition and pathogenesis of emphysema and fibrocystic disease of the lung with special reference to coal workers. Ann. N. Y. Acad. Sci. 200, 347.

63. Hilscher, W. and H. W. Schlipkoter. 1972. Antagonistic factors in the pathogenesis of coal workers' pneumoconiosis. Ann. N. Y. Acad. Sci. 200, 166.

64. Hinners, R. C., S. K. Burkhart and C. L. Punte. 1968. Animal inhalation exposure chambers. Arch. Environ. Health 16, 194.

65. Holma, B. 1969. Scanning electron microscopic observation of particles deposited in the lung. Arch. Environ. Health 18, 330.

66. Holt, P. F. 1957. Pneumoconiosis: Industrial Diseases of the Lung Caused by Dust. Edward Arnold Publishing Co., London. pp. 87-108.

67. Holt, P. F. and D. T. King. 1955. The chemistry of silica surfaces. J. Chem. Soc. 98, 773.

68. Holt, P. F. and C. W. Went. 1960. Studies on the nature of silicosis: a suggested mechanism of fibrogenesis. Brit. J. Industr. Med. 17, 25.

69. Holt. P. G., W. N. Bartholomaeus and D. Keast. 1974. Differential toxicity of tobacco smoke to various cell types including those of the immune system. Aust. J. Exp. Biol. Med. Sci. 52, 211.

70. Holt, P. G. and D. Keast. 1973. Acute effects of cigarette smoke on murine macrophages. Arch. Environ. Health 26, 300.

71. Holt, P. G. and D. Keast. 1977. Environmentally induced changes in immunological function: acute and chronic effects of inhalation of tobacco smoke and other atmospheric contaminants in man and experimental animals. Bac. Rev. 41, 205.

72. Hounan, R. F. 1954. Some reactions between inert dust and proteins. Arch. Industr. Hyg. Occup. Med. 9, 284.

73. Jacques, W. E., J. Coalson and A. Zervins. 1965. Application of the scanning electron microscope to human tissue. A preliminary study. Exp. Molec. Path. 4, 576.

74. Karnovsky, M. J. 1965. Formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell. Biol. 27, 137a.

75. Kavet, R. I. and J. D. Brain. 1977. Phagocytosis: quantification of rates and intercellular heterogeneity. J. Appl. Physiol. 42, 432.

76. Kessel, R. W., L. Monaco and M. A. Marchisio. 1963. The specificity of the cytotoxic action of silica, a study in vitro. Brit. J. Exp. Pathol. 44, 351.

77. Kettle, E. H. 1934. Infective pneumoconiosis: infective silicatosis. J. Path. Bac. 38, 201.

78. King, E. J. 1947. Solubility theory of silicosis: a critical study. Occup. Med. 4, 26.

79. King, E. J., G. P. Mohanty, C. V. Harrison and G. Nagelschmidt. 1953. The action of different forms of pure silica on the lung of rats. Brit. J. Industr. Med. 10, 9.

80. King. E. J., B. M. Wright, S. C. Ray and C. V. Harrison. 1950. Effect of aluminum on the silicosis producing action of inhaled quartz. Brit. J. Industr. Med. 7, 26.

81. Klosterkotter, W. and G. Buenmann. 1961. Animal experiments on the elimination of inhaled dust. In Inhaled Particles and Vapours Vol. 1. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 327-341.

82. Klosterkotter, W. and H. J. Einbrodt. 1967. Retention, penetration and elimination of inhaled dusts. In Inhaled Particles and Vapours Vol. 2. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 215-228.

83. LaBelle, C. W. and H. Brieger. 1961. Patterns and mechanisms in the elimination of dust from the lung. In Inhaled Particles and Vapours Vol. 1. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 356-368.

84. Lauweryns, J. M. 1970. The juxta-alveolar lymphatics in the human adult lung. Amer. Rev. Resp. Dis. 102, 877.

85. Lauweryns, J. M. 1971. The blood and lymphatic microcirculation of the lung. Pathol. Annu. 6, 365.

86. Lauweryns, J. M. and J. H. Baert. 1974. The role of the pulmonary lymphatics in the defenses of the distal lung: morphological and experimental studies of the transport mechanisms of intratracheally instilled particles. Ann. N. Y. Acad. Sci. 221, 244.

87. Lauweryns, J. M. and J. H. Baert. 1977. Alveolar clearance and the role of the pulmonary lymphatics. Amer. Rev. Resp. Dis. 115, 625.

88. Leiteritz, H., H. J. Einbrodt and W. Klosterkotter. 1967. Grain size and mineral content of lung dust of coal miners compared with mine dust. In Inhaled Particles and Vapours Vol. 2. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 381-392.

89. Leuchtenberger, C. and R. Leuchtenberger. 1971. The behavior of macrophages in lung cultures after exposure to cigarette smoke. In The Reticuloendothelial System and Immune Phenomena, Di Luzio, N. R. (ed.), Plenum Press, New York. pp. 347-360.

90. Levy, M. H. and E. F. Wheelock. 1975. Effects of intravenous silica on immune and non-immune functions of the murine host. J. Immunol. 115, 41.

91. Lotzova, E. and G. Cudk[illegible]. 1974. Abrogation of resistance to bone marrow grafts [illegible]silica particles. J. Immunol. 113, 798.

92. Lutz, W. 1967. Statistical methods as applied to immunological data. In Handbook of Experimental Immunology, Weir, D. M. (ed.), F. A. Davis Co., Philadelphia. pp. 1,163-1,206.

93. Macklin, C. C. 1950. The dust cells in the lungs of the albino mouse. Proc. Inst. Med. 18, 78.

94. Macklin, C. C. 1951. The dust cells in the lungs of the albino mouse: their structure, relation and mode of action. Lancet 1, 432.

95. Macklin, C. C. 1955. Pulmonary sumps, dust accumulations, alveolar fluid and lymph vessels. Acta. Anat. 23, 1.

96. Mancino, D. and N. Bevilacqua. 1977. Adjuvant effect of amorphous silica on the immune response to various antigens in guinea pigs. Int. Archs. Allergy Appl. Immun. 50, 97.

97. Marks, J. and G. Nagelschmidt. 1959. Study of the toxicity of dust with use of the in vitro dehydrogenase technique. Arch. Ind. Hyg. Occup. Med. 20, 383.

98. Martin, J. C., H. Daniel-Moussard, L. LeBouffant and A. Policard. 1972. The role of quartz in the development of coal workers' pneumoconiosis. Ann. N.Y. Acad. Sci. 200, 127.

99. Middlebrook, G. 1952. An apparatus for airborne infection of mice. Proc. of Soc. Biol. Med. 80, 105.

100. Miller, S. D. 1973. Effects of Carbon and Silica Dust on the Cellular and Humoral Immune Responses of Mice. M.S. Thesis, The Pennsylvania State University, 72 pp.

101. Miller, S. D. 1975. Experimental Silicosis: Effects on T and B Lymphocyte Immunocompetence and Macrophage Function. PhD. Thesis, The Pennsylvania State University, 122 pp.

102. Miller, S. D. and A. Zarkower. 1974. Alterations of murine immunologic responses after silica dust inhalation. J. Immunol. 113, 1,533.

103. Miller, S. D. and A. Zarkower. 1974. Effects of carbon dust inhalation on the cell-mediated immune response in mice. Inf. and Immun. 9, 534.

104. Miller, S. D. and A. Zarkower. 1976. Silica-induced alterations of murine lymphocyte immunocompetence and suppression of B lymphocyte immunocompetence: a possible mechanism. J. RES. 19, 47.

105. Moller, G. 1965. 19S antibody production against soluble lipopolysaccharide antigens by individual lymphoid cells. in vitro. Nature 207, 1,166.

106. Morgenroth, K., R. Blaschke and W. Schlake. 1973. Energy dispersive x-ray analysis of semi-thin sections in the scanning transmission. Beitrage zur Pathol. 150, 406.

107. Morris, T. G., W. H. Roberts, R. E. Silverton, J. W. Skidmore, J. C. Wagner and G. W. Cook. 1967. Comparison of dust retention in specific pathogen free and standard rats. In Inhaled Particles and Vapours Vol. 2. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 205-213.

108. Morrow, P. E. 1972. Lymphatic drainage of the lung in dust clearance. Ann. N. Y. Acad. Sci. 200, 46.

109. Nagelschmidt, G. 1960. The relation between lung dust and lung pathology in pneumoconiosis. Brit. J. Industr. Med. 17, 247.

110. Nagleschmidt, G., D. Rivers, E. J. King and W. Trevella. 1963. Dust and collagen content of lungs of coal workers with progressive massive fibrosis. Brit. J. Industr. Med. 20, 181.

111. Nash, T., A. C. Allison and J. S. Harington. 1966. Physico-chemical properties of silica in relation to its toxicity. Nature 210, 259.

112. Nourse, L. D., P. N. Nourse, H. Botes and H. M. Schwartz. 1975. The effects of macrophages isolated from the lungs of guinea pigs dusted with silica on collagen biosynthesis by guinea pig fibroblasts in cell culture. Env. Res. 9, 115.

113. Nowell, J. A., J. R. Gillespie and W. S. Tyler. 1971. Scanning electron microscopy of chronic pulmonary emphysema; a study of the equine model. IITRI/SEM. pp. 297.

114. Nowell, J. A., J. Pangborn and W. S. Tyler. 1970. Scanning electron microscopy of the avian lung. IITRI/SEM. pp. 231.

115. Nowell, J. A. and W. S. Tyler. 1971. Scanning electron microscopy of the surface morphology of mammalian lungs. Amer. Rev. Resp. Dis. 103, 313.

116. Nulsen, A., P. G. Holt and D. Keast. 1974. Cigarette smoking, air pollution and immunity: a model system. Inf. Immun. 10, 1,226.

117. Pearsall, N. N. and R. S. Weiser. 1968. The macrophage in allograft immunity. I. Effects of silica as a specific macrophage toxin. J. RES. 5, 107.

118. Pearsall, N. N. and R. S. Weiser. 1968. The macrophage in allograft immunity. II. Passive transfer with immune macrophages. J. RES. 5, 121.

119. Pernis, B. and F. Paronetto. 1962. Adjuvant effect of silica (tridymite) on antibody production. Proc. Soc. Exp. Biol. Med. 110, 390.

120. Pfefferkoen, G. E. 1970. Specimen preparation techniques. IITRI/SEM. pp. 89.

121. Policard, A. 1969. Defense of the lung against dust and its physiological basis. Arch. Environ. Health 1, 69.

122. Policard, A., A. Collet and L. Giltaire-Ralyte. 1957. Reticulohistiocyte reaction to silica: electron microscope study of fibrogenesis in the experimental silicotic granuloma. Bull. Hyg. 32, 583.

123. Robbins, S. L. 1974. Pathological Basis of Disease. W. B. Saunder Co., Philadelphia. pp. 509-515.

124. Rodnana, G. P., T. B. Benedek, T. A. Medsger and R. J. Cammarata. 1967. The association of progressive systemic sclerosis (scleroderma) with coal miners' pneumoconiosis and other forms of silicosis. Ann. Int. Med. 66, 323.

125. Ruttner, J., M. A. Spycher and H. Sticher. 1973. The detection of etiological agents in interstitial pulmonary fibrosis. Human Pathol. 4, 497.

126. Rylander, R. 1969. Alterations of lung defense mechanisms against airborne bacteria. Arch. Environ. Health 18, 551.

127. Rylander, R. 1971. Lung clearance of particles and bacteria. Effects of cigarette smoke exposure. Arch. Environ. Health 23, 321.

128. Rylander, R. 1973. Toxicity of cigarette smoke components: free lung cell response in acute exposures. Amer. Rev. Resp. Dis. 108, 1,279.

129. Rylander, R. 1974. Influence of infection on pulmonary defense mechanisms. Ann. N. Y. Acad. Sci. 221, 282.

130. Rylander, R. 1974. Pulmonary cell responses to inhaled cigarette smoke. Arch. Environ. Health 29, 329.

131. Rylander, R., M. C. Snella and I. Garcia. 1975. Pulmonary cell response patterns after exposure to airborne bacteria. Scand. J. Resp. Dis. 56, 195.

132. Sanders, C. L. and R. R. Adee. 1970. Ultrastructural localization of inhaled $^{239}PuO_2$ particles in the alveolar epithelium and in macrophages. Health Phys. 102, 823.

133. Schepers, G. H. W. 1955. Cytobiological manifestations of the surface properties of quartz. Arch. Industr. Health 12, 266.

134. Schiller, E. 1961. Inhalation, retention and elimination of dust from dogs' and rats' lungs with special reference to the alveolar phagocytes and bronchial epithelium. In Inhaled Particles and Vapours Vol. 1. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 342-347.

135. Schwartz, L. W., D. L. Dungworth, M. G. Mustafa, B. K. Tarkington and W. S. Tyler. 1976. Pulmonary responses of rats to ambient levels of ozone. Lab. Inves. 34, 565.

136. Schwartz, L. W., R. E. Kimball, M. Brummer and P. Lowrie. 1975. A comparative morphological study of ozone and oxygen induced pulmonary damage. Fed. Proc. 34, 839.

137. Selikoff, I. J., M. M. Key and D. H. K. Lee (eds.). 1972. Coal workers' pneumoconiosis. Ann. N. Y. Acad. Sci. 200, 1.

138. Spencer, H. 1968. The Pathology of the Lung. Pergamon Press, Philadelphia. pp. 376-402.

139. Spitznagel, J. K. and A. C. Allison. 1970. Mode of action of adjuvants: retinol and other lysosome-labilizing agents as adjuvants. J. Immunol. 104, 119.

140. Steel, R. G. and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., New York.

141. Suzuki, Y., J. Churg and T. Ono. 1972. Phagocytic activities of the alveolar epithelial cells in pulmonary asbestosis. Amer. J. Pathol. 69, 373.

142. Tobin, C. E. 1957. Human pulmonary lymphatic: an anatomic study. Anat. Rec. 127, 611.

143. Ulrich, L. 1963. Investigation of the effects of crystalline and amorphous silicic acid on cultures of lung tissue. Ann. Occup. Hyg. 6, 235.

144. U. S. Dept. of Health, Education and Welfare. 1974. Occupational exposure to crystalline silica. HEW PUB. No. (NIOSH) 75-120.

145. Vigliani, E. C., A. Boselli and L. Pecchiai. 1950. Pathology of blood serum in silicosis. Med. Lavoro. 41, 33.

146. Vigliani, E. C. and B. Pernis. 1959. An immunological approach to silicosis. J. Occup. Med. 1, 319.

147. Vigliani, E. C. and B. Pernis. 1961. The pathogenesis of silicosis: a review. Bull. Hyg. 36, 1.

148. Vigliani, E. C., B. Pernis and L. Monaco. 1961. A study of the action of quartz particles on macrophages in vitro. In Inhaled Particles and Vapours Vol. 1. Davies, C. N. (ed.), Cambridge University Press, Oxford. pp. 348-355.

149. Wagner, W. D., D. A. Fraser, P. G. Wright, O. J. Dobrogorski and H. E. Stokinger. 1968. Experimental evaluation of the threshhold limit of cristobalite-calcined diatomaceous earth. Amer. Ind. Hyg. Asso. J. 29, 211.

150. Wang, N. and W. M. Thurlbeck. 1970. Scanning electron microscopy of the lung. Human Pathol. 1, 227.

151. Weissbecker, L., R. D. Carpenter, P. C. Luchsinger and T. S. Osdene. 1969. In vitro alveolar macrophage viability. Effect of gases. Arch. Environ. Health 18, 756.

152. Weller, W. and W. T. Ulmer. 1972. Inhalation studies of coal-quartz dust mixture. Ann. N. Y. Acad. Sci. 200, 142.

153. Wilkinson, P. C. and R. G. White. 1966. The role of mycobacteria and silica in the immunological response of the guinea pig. Immunol. 11, 229.

154. Woldseth, R. 1973. X-ray Energy Spectrometry. Kevex Corporation, Pub., Burlingame, California. p. 4.7.

155. Wright, G. W. 1961. Structure and function of respiratory tract in relation to infection. Bac. Rev. 25, 219.

156. Zaidi, S. H. 1963. Experimental Pneumoconiosis. The Johns Hopkins Press, Baltimore.

157. Zarkower, A. 1972. Alterations in antibody response induced by chronic inhalation of O_2 and carbon. Arch. Environ. Health 25, 45.

158. Zarkower, A. and W. Morges. 1972. Alteration in antibody response induced by carbon inhalation: a model system. Inf. Immun. 5, 915.

159. Ziskind, M., R. N. Jones and H. Weill. 1976. Silicosis. Amer. Rev. Resp. Dis. 113, 643.