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Journal of Aerosol Medicine
Deposition, Clearance, and Effects in the Lung

The official journal of the International Society for Aerosols in Medicine

Symposium on Pharmaceutical Aerosols

Sponsored by the American Association for Aerosol Research (AAAR)
and
the North American Chapter of
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to be held during
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The object of this symposium is to provide overviews of and detailed insights into the function, generation, delivery and evaluation of pharmaceutical aerosols. Invited speakers will present extended reviews of selected topics that include background information and authoritative state-of-the-art summaries. Other authors of talks and posters will address specific topics in narrower scope and greater detail. Topics will include a multi-disciplinary analysis of drug delivery via aerosols, applications of pharmaceutical aerosols, drug delivery and site selection, diagnostic aerosols, aerosol delivery systems, non-CFC propellants, metering and dispersion of dry powder aerosols, prediction of respiratory deposition from in vitro and in vivo measurements, modeling of gas flow and aerosol deposition in the respiratory tract, modeling of lung geometry and aerosol deposition in the alveolar region, clinical applications and comparisons of aerosol therapies, and future challenges in the uses of pharmaceutical aerosols.

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Journal of Aerosol Medicine
Deposition, Clearance, and Effects in the Lung

Volume 3 Supplement 1 1990

Proceedings of the Symposium on Particle-Lung Interactions: "Overload" Related Phenomena

Rochester, New York, May 17 and 18, 1990

An authoritative bimonthly dealing with methods of diagnosis and treatment of pediatric patients with HIV infection...

Pediatric AIDS and HIV Infection

Fetus to Adolescent

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JOURNAL OF AEROSOL MEDICINE
Volume 3, Supplement 1, 1990
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Preface

GÜNTER OBERDÖRSTER and JURAJ FERIN

Over the past decade it has become increasingly clear that chronic inhalation studies performed with high exposure concentrations of highly insoluble particles leading to excessive particulate lung burdens will result in a range of adverse pulmonary effects, including lung fibrosis and even lung tumors. Most of these studies have been performed in rats and the term "Lung Overload" was coined for such situations, indicating effects which are not specific for a certain particle type. Since those effects were demonstrated for rather "inert" particles such as TiO_2,but, on the other hand, are also induced as specific effects by toxic particulate compounds, the terms "inert," "innocuous" or "nuisance" particles should not be used routinely to characterize the low toxicity particles. A continuum ranging from highly toxic particles such as quartz to particles with very low toxicity such as TiO_2 may exist; consequently, new terms for particles with low intrinsic toxicity have been introduced by regulatory agencies: Particles Not Otherwise Classified (PNOC) or Particles Not Otherwise Regulated (PNOR).

Besides these regulatory aspects, a number of scientific issues related to "Lung Overload" dealing with cellular events, underlying mechanisms, dosimetry and extrapolation to humans have been raised. This created a need to discuss these and other particle overload related issues in an international forum of scientists with expertise in different fields of inhalation toxicology, cellular biology, toxicokinetics and human health. Consequently, the North American Chapter of ISAM and the Environmental Health Sciences Center of the University of Rochester organized a symposium in Rochester, New York, on May 17 and 18, 1990 on "*Particle-Lung Interactions: 'Overload' Related Phenomena.*" Specific questions were addressed in invited presentations by twelve expert scientists followed by intensive discussions with eighteen invited panelists and the audience. The total attendance at the symposium was 142 from 10 countries. Obviously, a symposium of this structure and scope would not have been possible without the financial support of a number of sponsors. The generous financial assistance of the following companies and agencies is greatly appreciated:

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We also wish to acknowledge the valuable advice of the Scientific Advisory Committee (Arnold Brody, Kevin Driscoll, Timothy Gerrity, Marvin Kuschner, Bruce Lehnert, Joe Mauderly, Hartwig Muhle, David Warheit, and Ron Wolff) and the enthusiastic efforts of the Local Organizing Committee (Jacob Finkelstein, Paul Lambiase, Robert Mermelstein, Paul Morrow, Sidney Soderholm and Mark Utell).

The symposium was organized in four sessions; the first three were devoted to cytological, cell biological and toxicokinetic aspects of particle-cell interactions and the fourth to implications for research and human health. After each session, four to five panelists and the audience discussed specific questions raised by the session chairpersons and the audience. A symposium summary concluded the intensive discussions of the two-day meeting

This supplementary issue of the Journal of Aerosol Medicine contains the peer-reviewed papers of the invited speakers, grouped by session topic, and a summary paper by R. O.McClellan. Although basic observations such as the functional impairment of the alveolar macrophages, particle translocation and epithelial cell proliferation in a particle overload condition are firmly established, fundamental events at a cellular and molecular level leading to the pathological outcome of particle overload in the lung are not well delineated and additional data are only now forthcoming. Perhaps future studies on mechanisms underlying events occurring after low level exposure to toxic particles such as quartz or even asbestos fibers will give some clues about the nature of these events in a particle overload condition with particles of low toxicity. We need to know more about cell-cell interactions *via* released mediators; we need to understand better the mechanisms involved in lung particle clearance and we need to advance our knowledge of basic mechanisms leading to chronic lung injury such as pulmonary fibrosis and lung tumors in order to assess better the significance of particle-lung interactions in the human lung. Does the human respiratory tract respond in the same way as that of the rat to high particulate lung burdens? Is the lung pathology observed in some heavily exposed coal miners a consequence of overload? Since not all heavily exposed workers will eventually show chronic lung injury might there be a delicate balance between pro-mitogenic and anti-mitogenic mediators released from affector cells? Is one consequence of a "physical overload" in the lung - hypothesized by P.E. Morrow as a volumetric overload of alveolar macrophages (*Fund. Appl. Tox.* **10**: 369-384, 1988) - the generation of a state of "biochemical overload?"

Questions like these were addressed at the symposium, and while no final answers could be given for many, the enthusiastic response to the symposium and the extensive discussions clearly showed that the symposium dealt with a timely and important area of pulmonary research. The Rochester symposium was a first attempt to bring together people from academe, research institutions, industry and regulatory agencies to discuss the topic of lung overload from a wide variety of viewpoints. This will undoubtedly be followed by other specialized symposia and workshops in the future. We thank the invited speakers, panelists, chairpersons and attendees for their interest and active participation in the symposium.

Günter Oberdörster
Juraj Ferin
Symposium Co-Chairmen

JOURNAL OF AEROSOL MEDICINE
Volume 3, Supplement 1, 1990
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Abstracts of Presentations

Lehnert B.E.: Alveolar Macrophages in a Particle "Overload" Condition

Alveolar macrophage (AM)-mediated particle clearance from the lung via the conducting airways is an important mechanism by which relatively insoluble particles are translocated from the alveolar region. Diminution in the kinetics of removal of particles from the lung following the deposition of excessive particulate lung burdens (particle "overload"), which can be functionally viewed as the emergence of a sequestration compartment(s), suggests AM-related bases inasmuch as these cells are usually the primary reservoirs of deposited particles. Specific details about the mechanisms involved in AM-mediated lung clearance or about processes that may favor particle retention in the lung have not been well delineated. In the present investigation, the retention kinetics of a low to a relatively high lung burden of uniform polystyrene microspheres was examined with the highest burden studied resulting in a condition of particle "overload". We also assessed the lung's free cell response to these burdens over a prolonged period following the intrapulmonary deposition of the particles as we concurrently investigated particle-AM relationships during the alveolar clearance of the different lung burdens. Evidence obtained suggests the particles were gradually redistributed among members of the lung's free cell population of phagocytes during alveolar clearance. Additionally, polymorphonuclear leukocytes and blood monocyte-, pulmonary interstitial macrophage-like cells became increasingly prominent in the lung's free cell population over time during a condition of particle overload with the polystyrene microspheres; such findings suggest that these cells may potentially play a pathogenic role when lung burdens are excessive. Electron microscopic analyses suggested that aggregates of particle-laden macrophages, particle-containing Type I pneumocytes, and particle-containing pulmonary interstitial macrophages represent particle sequestration sites that contribute to diminished lung clearance during particle overload. However, our analyses of particle-AM relationships during particle overload point to AM with particulate volume loads equal to or in excess of 60% of their normal volumes as being the main sequestrating compartment to which diminished rates of lung clearance can be virtually totally attributed. Moreover, the size of the AM-particle sequestering compartment appeared to remain stable over a 5 month period after the particles were deposited in the lungs. This latter observation suggests that once developed, the AM-particle sequestration compartment is essentially irreversibly maintained.

Adamson I.Y.R.: Cellular Responses and Translocation of Particles Following Deposition in the Lung

Instillation of carbon into mouse lung results in a rapid increase in cells recovered by bronchoalveolar lavage. Initially the increase is due to

polymorphonuclear leukocytes (PMN), then after 12 hours, alveolar macrophage (AM) numbers increase and reach a maximum at 2-3 days. Whereas the initial increase in AM is due to migration of monocyte-derived cells, after 1 day AM numbers are maintained by proliferation and migration of interstitial cell precursors. In a particle overload situation, the number of AM recovered at 1 day peaked with a 1.0 mg dose and did not increase as the dose was raised though the duration of the maximal response was extended. At high levels, translocation of particles into lung parenchyma was seen and carbon was found in Type 1 epithelial cells, in interstitial macrophages (IM) and in hilar lymph nodes. An alveolar overload situation was induced by reducing phagocytosis and clearance. We instilled carbon to the lungs of mice depleted of leukocytes by whole body irradiation. The usual eflux of PMN and AM was delayed and reduced, leading to greater particle transfer to the interstitium and lymph nodes than after carbon alone. When silica was injected to irradiated mice, the increase in PMN and AM was reduced, and many particles reached the IM. At 16 weeks radiated mice that received silica had a higher weight of retained particles in the lungs, and collagen measurements were much higher than after silica or irradiation alone. The results suggest that alveolar overload greatly enhances particle translocation to the interstitium where secretion of any macrophage-derived factors is more likely to be effective in fibroblast stimulation.

Rom W.N., Churg A., Leapman R., Fiori C. and Swyt, C.: Particle Burden in Alveolar macrophages of Nonsmoking Individuals Occupationally Exposed to Inorganic Dusts

Alveolar macrophages recovered by bronchoalveolar lavage from individuals with occupational inorganic dust exposure are laden with particles. We evaluated 42 non-smoking males with long-term exposure to asbestos (27), coal (7), or silica (8), and normals (8) to determine a particle burden per 10^6 alveolar macrophages. Scanning/transmission electron microscopy and energy-dispersive x-ray analysis were utilized to evaluate the particles following bleach digestion of the cells, or of alveolar macrophage sections. There was a four-fold ($p<0.01$) increase in the number of particles in the dust-exposed. There was also a striking increase in silica particle number in the silica-exposed ($p<0.02$) but not in the other dust-exposed groups. One-third of the coal miner's cells contained silica particles predominantly <0.5 µm. In the asbestos-exposed, there was one chrysotile fiber per 35 cells, and one amosite fiber per 215 cells consistent with the known mixed exposure of workers exposed to insulation products in the United States. No crocidolite was observed in any of the cells and tremolite was identified in two controls and two workers. Computer-generated maps of elements comprising the particles demonstrated the in situ localization of the particles and identified many very small alumino-silicates, particularly in coal miners. Particle analysis is a useful technique to evaluate type and amount of exposure, to evaluate alveolar clearance, and may be useful to investigate macrophage activation.

Last J.A., Wu R., Chen J, Gelzleichter T, Sun W-M and Armstrong L.G.: *Particle-Cell Interactions: Lung Fibrogenesis*

Many inhaled particulates can cause a fibrotic response of the lung. Lung fibrosis, whether defined pathologically, biochemically, or physiologically, may occur in either an acute or chronic time frame after the fibrogenic insult. Several biochemical changes in lung collagen are associated with the later stages of this response. These include increased amounts of total lung collagen, a change in the ratio of type I to type III collagen (which may occur in response to some, but not all, fibrogenic stimuli), and changes in the

relative content of hydroxylysine and of hydroxylysine-derived cross-links in fibrotic lung collagen.

Early events after initial exposure to a fibrogenic agent include acute lung injury, cell damage or cell death, lung edema, and lung inflammation. The relationship between these early events and the eventual outcome, lung fibrosis, is an area currently under very active investigation. It is also probably the component of the lung's response to injury that we least understand. A complex array of mediators and factors have been, and are being, described that may modulate the biochemical interactions and communication between cells in the lung. The possible role of such signal molecules in relating early lung damage to subsequent irreversible structural and functional alterations of the lung has not yet been defined. Many investigators assume that early and late events are linked by signal molecules that cause the selection of specific subpopulations of mesenchymal fibroblasts with enhanced proliferative activity and/or altered phenotypic expression of collagen synthesis.

Mossman B.T., Janssen Y.M.W., Marsh J.P., Manohar M., Garrone M., Shull S. and Hemenway D.: Antioxidant Defense Mechanisms in Asbestos-Induced Lung Disease

Several studies suggest that active oxygen species (AOS) are involved in the development of asbestos-induced lung diseases. Experiments in this laboratory have focused on antioxidant enzymes as preventive agents of asbestos-induced cell injury when added to cultures of alveolar macrophages (AMs), tracheobronchial epithelial cells, the progenitor cells of lung cancer (bronchogenic carcinoma), and lung fibroblasts, a cell type affected in asbestosis. Most recently, lung injury, inflammation, and pulmonary fibrosis have been ameliorated in an inhalation model of asbestosis using administration of antioxidant enzymes to rats during their exposure to asbestos. Current studies are focusing on the patterns and mechanisms of induction of antioxidant enzymes in the lung after inhalation of asbestos. These studies indicate that levels of antioxidant enzymes [total superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase] are increased in lungs within days after the initiation of exposure to high airborne concentrations (~ 7 mg/m^3 air) of crocidolite asbestos. Use of cDNA probes for CuZn and Mn-containing SODs indicates that steady-state mRNA levels of Mn-SOD are increased in the lungs of asbestos-exposed animals, while CuZn-SOD expression is unchanged. Results suggest that inhalation of crocidolite asbestos induces increased expression of an antioxidant enzyme in lung which is induced by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF) in a variety of cell types.

Driscoll K.E., Maurer J.K. and Crosby L.L.: Overload of Lung Clearance Is Associated With Activation of Alveolar Macrophage Tumor Necrosis Factor and Fibronectin Release

This report summarizes recent findings on the relationships among overloaded lung clearance, activation of alveolar macrophages (AM) release of inflammatory mediators and the development of fibrosis using TiO2 as a model nuisance type dust. Briefly rats were intratracheally instilled with 2-100 mg TiO2/kg body weight and AM tumor necrosis factor or fibronectin release determined ex vivo 1, 7, 14 and 28 days after exposure. Lung dust burdens were determined 1 and 28 days after exposure. Histopathology was assessed 28 and 90 days after exposure. Intratracheal instillation of ≥50 mg/kg TiO2 resulted in overloaded lung clearance. TiO2 doses ≥50 mg/kg stimulated transient increases in AM TNF release and a persistent increase in AM fibronectin secretion.

Histopathology demonstrated dose-related interstitial inflammation with fibrosis, developing only after treatment with ≥50 mg/kg TiO2. Results from these studies suggest activation of AM secretory activity may play a key role in adverse pulmonary responses to high dust burdens of relatively innocuous materials. Studies investigating in vitro responses of AM to dust indicated that direct TiO2:AM interaction does not stimulate release of TNF or fibronectin, however, pre-exposure to γ-interferon can render AM responsive to TiO2 with respect to increased TNF release.

Kreyling W.G.: Interspecies Comparison of Lung Clearance of "Insoluble" Particles

Lung clearance studies after the inhalation of monodisperse, radiolabelled test particles including lung retention measurements and excretion analysis allow for estimates of the kinetics of long-term particle transport out of the thorax into the gastro-intestinal tract. Data of several interspecies comparisons using either radiolabelled fused aluminosilicate particles or $^{57}Co_3O_4$ particles were reviewed and compared. Species included were: man, baboon, beagle dog, guinea pig, HMT rat, F-344 rat, Long-Evans rat, hamster, mouse.

Particle transport M(t) after the first days after inhalation is a slow clearance mechanism which is independent of the particle material and size used (0.5 - 4 μm geom. diameter). M(t) was reproducible in the experimental species studied. In man, baboon, and dog the initial daily fraction M_0 of the contemporary lung burden transported out of the thorax is 0.001 d^{-1} which is an order of magnitude less than the initial rates in rodents. Particle transport rate decreases rapidly from its initial value in all species studied. The decay of particle transport varies considerably between the species and strains. The half-life of the decreasing transport rate is slower in man, dog, F-344 rat, hamster and mouse (100 - 200 days) than in baboon, HMT rat and Long-Evans rat (< 50 days). From these studies estimates of lung retention during chronic aerosol exposure showed no equilibrium value indicating that long-term particle transport is not a sufficiently effective clearance mechanism to keep the lung burden from continuousy increasing during chronic exposure.

Muhle H., Creutzenberg, Bellman B., Heinrich U. and Mermelstein R.: Dust Overloading of Lungs: Investigations of Various Materials, Species Differences and Irreversibility of Effects

In separate inhalation investigations, rodents (Wistar rats, Fischer-344 rats, Syrian golden hamsters, NMRI and C57BL mice) were exposed to various dusts such as test toner (polymer pigmented with carbon black), polyvinyl chloride, carbon black, diesel exhaust and two crystalline forms of titanium dioxide (anatase and rutile). The animals inhaled various concentrations (0.8 to 64 mg/m^3) of these particles for up to 2 years.
Alveolar clearance retardation was detectable above a retained pulmonary burden of 0.5 mg per rat lung, and a substantial decrease in the clearance rate (about a factor of 6) was observed following heavy dust loading, exceeding 10 mg dust per rat lung. Above a threshold lung burden, signs of lung overloading persisted 15 months after cessation of exposure in F-344 rats. Retardation of alveolar clearance was also observed in hamsters, commencing at higher lung burdens (normalized to lung weight) than in rats. At high dust exposure levels, persistent pulmonary inflammation was present in both species. In rats the concentration of lavagable cells remained constant, with decreased macrophages and increased polymorphonuclear neutrophils (PMN) noted, while in hamsters, the cell count increased substantially in both macrophages and

PMN's. A retarded particle clearance was also observed in mice at a lung burden above 1 mg/lung.
These results, accompanied by published accounts, indicate that the lung overloading phenomenon is noted among a variety of species and materials. It is generally observed upon exceeding a threshold lung burden with particles of low solubility and low acute toxicity for considerable periods of time.

Stöber W., Morrow P.E., Morawietz G., Koch W. and Hoover M.D.: Developments in Modeling Alveolar Retention of Inhaled Insoluble Particles in Rats

The development of a non-linear, physiology-oriented compartmental kinetics model of clearance and retention of insoluble particles in the alveolar region of rat lungs is described. The model recognizes the dominant role of the alveolar macrophages in alveolar clearance and retention and assumes that, eventually, increasing burdens of phagocytized particles impair the mobility of alveolar macrophages. Thus, the macrophage-mediated particle removal process will be retarded. In continuous inhalation exposures, this may cause the sequestration of heavily loaded macrophages and a general overloading of the alveolar region of the lung with retained particles. A basic model design accounting for macrophage life time, phagocytosis rate, mobility decline and limited load capacity was applied for the simulation of experimental data of several chronic and subchronic inhalation studies. The results were very good, but some of the model parameters did not comply with the self-imposed quality criteria. Apparently, the average load of the macrophage pool was an insufficient parameter to account consistently for sequestration. The model was then revised and features now particle load distributions in the macrophage pool. Preliminary efforts to simulate the same experimental inhalation data as before gave again very good results and the model parameters utilized did no longer show the previous inconsistencies.

Lippmann M. and Timbrell, V.: Particle Loading in the Human Lung - Human Experience and Implications for Exposure Limits

Timbrell's analyses of fiber burdens in the post-mortem lungs of workers with long-term inhalation exposures to a variety of amphiboles have shown that the clearance of fibers is strongly dependent on lung burden and its associated lung fibrosis, with a small percentage of very heavily exposed workers having little, if any clearance from parts of the lung. The extent of lung fibrosis is proportional to the total surface of retained mineral particles for both fibers and more compact particles. The human data base from the asbestos workers can provide a sound basis for the development of more generic models describing the influence of lung burden of mineral dust on particle deposition in, and clearance from, human lungs. The implications of the results obtained to the pathogenesis of chronic lung diseases and the evaluation and/or establishment of exposure limits are also discussed, along with some research needs to facilitate interspecies extrapolation of fiber toxicity data.

Mauderly J.L., Cheng Y.S. and Snipes M.B.: Particle Overload in Toxicological Studies: Friend or Foe?

The overloading of particle clearance is an important issue in the design and interpretation of inhalation toxicological studies. This issue is particularly important in chronic inhalation bioassays in rats, in which overloading is associated with inflammation, epithelial proliferation, and fibrosis, which may amplify carcinogenic responses or, as suggested by some, even induce cancer regardless of the inhaled material. At present, the key issue is whether or not data from exposures causing overload in animals are

useful for predicting health effects in man. A review of reports of chronic inhalation studies in rats exposed to a spectrum of materials suggests that not all exposures resulting in overloading cause cancer, and that the cancer incidences from exposures causing overloading appear to reflect the relative carcinogenic potentials of the test materials. Data from such exposures, however, do little to establish the exposure-response relationship at lower doses most typically relevant to human exposures. Responses observed under overload conditions may be relevant to responses of humans exposed to high (occupational) levels of dusts, of humans with clearance impairments, or of humans in whom inflammation, epithelial proliferation, or fibrosis are concurrently induced by other agents. We need to know more about the relative contributions of carrier particles and particle-borne carcinogens to carcinogenicity under overloaded and non-overloaded conditions. We need to know more about the function of retained particles as reservoirs of particle-borne carcinogens. We need to know more about the potential amplification of carcinogenic responses at low doses by clearance impairments and inflammatory, proliferative, and fibrotic responses induced by other agents. Most importantly, we need a better understanding of the mechanisms of carcinogenesis. At this time, we have sufficient ability to design animal studies to either include or avoid overload. It is concluded that it may be useful to include at least a "minimal overload" level in inhalation bioassays of poorly-soluble particles, and that this approach might be a useful substitute for the classical maximum tolerated dose in setting exposure limits.

Witschi H.: Lung Overload: A Challenge for Toxicology

Chronic lung overload may result in the development of fibrosis and of tumors in the lung parenchyma. The essential question that must be answered is whether there exists a threshold of exposure below which these effects are unlikely to occur. Both threshold and non-threshold mechanisms appear to exist for the two conditions, as illustrated by selected examples. Definition of a threshold is often driven by present analytical approaches. Mechanistic studies should not only address development of lesions, but also examine events that determine tissue recovery.

McClellan R.O.: Particle Overload in the Lung: Approaches to Improving our Knowledge

Lung overload is a condition characterized by (1) an overwhelming of normal clearance processes under certain exposure conditions, (2) resulting in lung burdens greater than predicted from disposition kinetics observed at low exposure concentrations, (3) with associated pathophysiological changes including altered macrophage function, inflammation and pulmonary fibrosis and (4) an uncertain association with an increased incidence of lung tumors in studies conducted in rats.

Our present knowledge is not sufficient to distinguish between the role of compound specific mechanisms and non-compound specific mechanisms in the development of the lung overload condition. This is of particular concern when assessing the potential human health risks of exposure to particles using information from inhalation studies conducted in rats. An improved knowledge base on this issue can be developed through appropriately designed and interpreted studies.

This paper (a) reviews the role of studies with an exposure-dose-response orientation conducted at multiple levels of biological organization in understanding and assessing human health risks for airborne particles, (b) discusses the overload condition with particular reference to understanding compound specific versus non-specific effects of inhaled materials, and (c) recommends approaches to the conduct and interpretation of inhalation studies with particulate materials conducted in rats.

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Alveolar Macrophages in a Particle "Overload" Condition

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ABSTRACT

Alveolar macrophage (AM)-mediated particle clearance from the lung via the conducting airways is an important mechanism by which relatively insoluble particles are translocated from the alveolar region. Diminution in the kinetics of removal of particles from the lung following the deposition of excessive particulate lung burdens (particle "overload"), which can be functionally viewed as the emergence of a sequestration compartment(s), suggests AM-related bases inasmuch as these cells are usually the primary reservoirs of deposited particles. Specific details about the mechanisms involved in AM-mediated lung clearance or about processes that may favor particle retention in the lung have not been well delineated. In the present investigation, the retention kinetics of a low to a relatively high lung burden of uniform polystyrene microspheres was examined with the highest burden studied resulting in a condition of particle "overload". We also assessed the lung's free cell response to these burdens over a prolonged period following the intrapulmonary deposition of the particles as we concurrently investigated particle-AM relationships during the alveolar clearance of the different lung burdens. Evidence obtained suggests the particles were gradually redistributed among members of the lung's free cell population of phagocytes during alveolar clearance. Additionally, polymorphonuclear leukocytes and blood monocyte-, pulmonary interstitial macrophage-like cells became increasingly prominent in the lung's free cell population over time during a condition of particle overload with the polystyrene microspheres; such findings suggest that these cells may potentially play a pathogenic role when lung burdens are excessive. Electron microscopic analyses suggested that aggregates of particle-laden macrophages, particle-containing Type I pneumocytes, and particle-containing pulmonary interstitial macrophages represent particle sequestration sites that contribute to diminished lung clearance during particle overload. However, our analyses of particle-AM relationships during particle overload point to AM with particulate volume loads equal to or in excess of 60% of their normal volumes as being the main sequestrating compartment to which diminished rates of lung clearance can be virtually totally attributed. Moreover, the size of the AM-particle sequestering compartment appeared to remain stable over a 5 month period after the particles were deposited in the lungs. This latter observation suggests that once developed, the AM-particle sequestration compartment is essentially irreversibly maintained.

INTRODUCTION

Alveolar macrophages (AM) are widely recognized as an important first-line cellular defense mechanism against inhaled particles that deposit in the alveolar region of the lung. Such defense

Key words: Lung clearance, lung retention, particle overload, lavage, alveolar macrophages, particle transport, particle redistribution.

is initially afforded by the relatively rapid phagocytosis of deposited particles by the AM, which serves to prevent particle interactions with the alveolar extracellular lining fluid and alveolar epithelial cells. In addition to this cellular compartmentalization, several lines of evidence also indicate that the AM play a major role in the removal of particles from the lung mainly via their physical translocation up the conducting airways with their contained particulate burdens (Lehnert and Morrow, 1985; Sorokin and Brain, 1975; Gibb and Morrow, 1962).

Although mechanisms involved in the process of phagocytosis of particles by macrophages are now relatively well understood, details of mechanisms involved in the AM-mediated particle clearance process or about AM-related mechanisms that may favor the retention of particles in the lung have not been well delineated. Using the rat model and initial lung burdens of noncytotoxic polystyrene microspheres that ranged from ~86 μg to ~3.7 mg, we set out in the present study to obtain information about these processes by analyzing particle-AM relationships over the course of alveolar clearance of the different lung burdens of particles, the highest burden of which brought about a condition of particle overload. We present evidence herein: 1) that particles are gradually redistributed among the lung's AM population during alveolar clearance, 2) that, consistent with Morrow's original hypothesis (1988), a cessation of AM removal from the lung in a particle overload condition occurs when the volumetric loads of particles in AM are equivalent to or exceed ~60% of their normal volumes, and 3) that once developed, the size of the AM-particle sequestration compartment, which appears to essentially totally account for the particle overload condition, is persistently stable for a prolonged period after particle deposition in the lung.

MATERIALS and METHODS

Animals and Particle Instillations

Male Fischer-344 rats (250-275g, SPF, Harlon Sprague Dawley, Indianapolis, IN) maintained in a barrier facility were used in this study. One group of rats was intratracheally instilled with 0.4 ml of sterile phosphate buffered saline (PBS, pH 7.3) under Ethrane® anesthesia, as previously described (Lehnert *et al*, 1985). A second group was intratracheally instilled with 0.4 ml PBS containing ~86 μg (1.6×10^7) prewashed (Lehnert *et al*, 1985) fluorescent, carboxylated, polystyrene microspheres (2.13 μm dia., Polysciences, Inc., Warrington, PA). A third group of rats was instilled with ~1 mg (2×10^8) of the microspheres, and a fourth group of animals was intratracheally instilled with with ~3.7 mg (6.8×10^8) of the microspheres. Hereafter, the PBS-instilled animals will be referred to as the control group, and the rats that were administered the 86 μg, 1 mg, and 3.7 mg initial lung burdens will be referred to as the low burden (LB), the medium burden (MB), and the high burden (HB) groups, respectively.

Bronchoalveolar Lavage and Lung Free Cells

Rats were sacrificed at the indicated post-instillation times by I.P. injection of 50 mg pentobarbital sodium. The animals were exsanguinated and the trachea of each rat was cannulated with a blunt, 18-gauge needle secured with ligature and the trachea and lungs were excised *en bloc*. The lungs were lavaged by two consecutive series of lung washings (lavage series 1 and 2). Common to each series, the lungs were washed six times with 8 ml of room temperature PBS while they were gently massaged. Fluids retrieved during each series were pooled in separate tubes maintained in ice. Lungs were excluded from the study when excessive leakage occurred during the lavage procedure, i.e., <90% of the instilled volume was recovered.

The total numbers of cells harvested by each lavage series were counted with a hemocytometer. Cytocentrifuged slide preparations (Shandon Southern Cytospin, Shandon Southern Products, Lts., Cheshire, UK) were stained with Diff Quik® stain (American Scientific Products, McGaw Park, IL) for cell differential analyses and particle-cell analyses (to be described). A minimum of 300 cells per lavage series was assessed for cell types. Because the percentages of the cell types in the lavage series 1 and 2 samples from any given lung were closely similar, if not identical (data not shown), the cell differential values obtained from the two wash series were averaged. These values, in conjunction with the cumulative numbers of cells harvested by the two lavage series, were then used to calculate the total numbers of each cell type obtained.

Analysis of Particles in the Lavaged Cell Samples

Particle-cell relationships among the cells harvested from the LB group were assessed by light microscopy under oil immersion optics (630x), as previously described (Lehnert *et al*, 1989). Briefly, one thousand to two thousand cells per lavage series were observed in random sequential fields, and the number of microspheres within each cell type was counted. A different approach was used to quantitate particle burdens in the lung free cells from the MB and the HB groups (Lehnert *et al*, 1990). With these samples, a minimum of 300 AM per lavage series were initially assessed for the percentages of the AM that contained the microspheres. Then, randomly selected fields of particle-containing cells were viewed using a Zeiss Ultraphot microscope. The cell profiles and microspheres were visualized using a combination of transmitted tungsten light and fluorescence microscopy (excitation: 450-490 nm, emission: 510-520 nm). The particle burdens in cells with relatively few microspheres, e.g., 1-10 particles, were counted by direct visualization. Those cells that contained larger burdens of microspheres were quantitated indirectly by first increasing the microscope's magnification and then imaging the cells on a high resolution black-and-white (B-W) monitor where the microspheres could be more easily counted. The B-W image was produced using a CCD video camera that was mounted on the microscope and attached to an image analyzer (Dapple Systems, Sunnyvale, CA). The heavily loaded cells appeared to contain the microspheres piled on top of one another in layers after cytocentrifugation, an effect that was confirmed by scanning electron microscopy. In these cases, the numbers of particles in the cells were estimated by counting the microspheres at different planes within the cells. This differential focus method was facilitated by the narrow depth of focus on the higher magnification objectives and the magnified (7x) CRT image. The above procedures were used: 1) to quantitate the particles in a minimum of 150 particle-containing AM in the lavage series 1 and 2 samples from each lung, and 2) to quantitate the numbers of microspheres in a minimum of 100 PMN in each lavage series sample when these cells contained the microspheres, i.e., following the instillation of the HB (to be discussed). Pilot studies of AM lavaged from the lungs of rats instilled with 4×10^8 microspheres showed that the particle burdens in the AM ascertained by the image analyzing system were virtually identical to those counted by careful manual microscopy (Lehnert *et al*, 1988). It should also be noted that scanning electron microscopic examinations of cytocentrifuged slide preparations made from various AM samples confirmed that the microspheres were virtually all contained (>99%) in the AM and that they were not merely adherent to the surfaces of AM.

Similar to our previously reported findings (Lehnert *et al*, 1985a), the results from the foregoing particle-cell analyses indicated that cells harvested during lavage series 1 and 2 were equally representative of all the lavaged cells with regard to their particle distributions; the lavage series 1 and 2 cells obtained from individual lungs were often identical in the percentages of cells that phagocytized the spheres as well as in the distributions of the microspheres among the cells that contained them. Given such consistent similarities, the particle cell data (i.e., the percentage of the cells with n particles, where n= 1, 2, 3..., etc., particles) from the lavage series 1 and 2 samples were averaged. These average values and the cell differential data, in conjunction with the lavaged cell number data, were used to calculate the total number of lavaged cells that contained a given burden of microspheres. Thereafter, the total numbers of lavaged cells in a given particle category were multiplied by the number of particles defining the category to determine the numbers of particles associated with the cells in the particle burden category. These numbers, in turn, were summed over all cell-particle burden categories in order to estimate the total numbers of particles lavaged.

Based on the numbers of particles lavaged from the lungs relative to the total numbers of particles retained in the lungs at each sacrifice time, and the assumption that all retained microspheres in the lungs were associated with free phagocytes, lavage recovery efficiencies (Lehnert and Morrow, 1985) of the lung free cells were calculated. The lavage recovery efficiencies for the particle-instilled rats were subsequently used in conjunction with the lavage extrapolation method (Lehnert and Morrow, 1985) to estimate the total sizes of the AM populations (and PMN when these cells contained particles) at each sacrifice time in order to further estimate the numbers of AM in the various particle categories over the course of alveolar clearance. Deviations from the assumption that all of the retained particles were exclusively contained in lung phagocytes during the clearance of the three lung burdens will be discussed later.

Preparation of Tracheobronchial Lymph Nodes and Lung Tissue for Particle Analyses

Three tracheobronchial lymph nodes that receive lymphatic drainage from the lung (Lehnert *et al*, 1986) were removed prior to excision of the trachea and lungs. The nodal tissues from each

animal were chemically dissolved in 10 ml of 10% potassium hydroxide (KOH) for 48 hr at ~60°C. The trachea and lungs were minced after lavage and the tissue fragments were similarly solubilized in 15 ml of the KOH. Preliminary studies revealed that equivalent incubations of the test microspheres in the KOH did not decrease their numbers or noticeably diminish their fluorescent characteristics. Moreover, when known numbers of microspheres were instilled into each of several lungs and the lungs were then immediately placed in the KOH solution and subsequently solubilized, total particle recovery was achieved.

Analyses of Lung and Tracheobronchial Lymph Nodal Particles

The solubilized lung and tracheobronchial lymph nodal fluids were quantitatively diluted with PBS, vortexed, and sonicated. Aliquots from the tissue samples were then passed through 25 mm dia.,1.0 μm pore size Nucleopore polycarbonate membrane filters (Nucleopore Corp., Pleasanton, CA) to collect the microspheres. A minimum of three filters were prepared for each tissue sample and the numbers of particles on the filters were estimated using the previously described image analyzer system. Fifteen fields per filter were randomly surveyed (magnification: 470x), and the average number of particles per field was calculated for the filter samples. The total number of particles on a filter was estimated by multiplying this number by a factor derived from the quotient of the total particle collection area on the filter and the area observed in one field of vision. The total number of particles in a given sample was then calculated from the total number of particles so counted from the filtered aliquots of known volumes and from the original volumes of the solubilized tissue samples.

Kinetics of Lung Retention of the Microspheres

The estimated numbers of particles lavaged from the lungs and the numbers of particles found in the lungs post-lavage were summed to estimate the total lung burdens of the microspheres. The lung burdens measured during the study were used for analyses of the lung retention characteristics of the three burdens of the microspheres initially instilled. Inspection of the particle retention data for each lung burden condition suggested that the retention characteristics of the microspheres in the lungs could be satisfactorily described by two-component, negative exponential equations of the following form: $LB_{(t)} = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$, where $LB_{(t)}$ is the lung burden present t days after instillation, and A and B are the coefficients associated with the retention rates λ_1 and λ_2, respectively. The lung retention data were fitted to the two-component model by nonlinear least squares (Draper and Smith, 1966); whereas two terms were definitely required to model each of the three sets of the data, the use of three terms for any of the three lung burdens gave no significant reduction in the sum of squares. The fitting of the lung burden data was accomplished by curve stripping (Fiserova-Bergerova, 1983), i.e., by fitting one exponential to the last two time points of the lung retention data and then holding the exponent of that term fixed while fitting the other three parameters. In addition, the curves were constrained to pass through the numbers of particles instilled on day zero.

We lost the microspheres in the day 86 HB lavaged lung samples due to a technical problem. Two methods were used to deal with this loss. The first method was to fit curves to the HB retention data without the day 86 lung burden data. With the second approach, the values for the retained lung burdens on day 86 were estimated from the numbers of particles lavaged from the lungs in conjunction with interpolated values for the lavage recovery efficiencies of the particles (Lehnert and Morrow, 1985), i.e., the fraction of the retained particles that are lavaged by a standardized lavage protocol with the interpolated values being derived from the lavage recovery efficiencies obtained on days 55 and 168 after particle deposition. Thereafter, the lung retention data, including the estimated day 86 retained lung burdens, were fitted to a two-compartment model, as previously described.

RESULTS

Lavaged Lung Free Cell Numbers and Types

On average, ~95% of the cells lavaged from the PBS and particle-instilled lungs, regardless of their initial burdens of microspheres, were AM at each post-instillation time. The numbers of AM lavaged from the lungs treated with the LB and MB of the particles were usually similar to those obtained from the control lungs, Figure 1. On the other hand, the numbers of AM harvested from the HB lungs were ~80% and ~45% higher than control values at the 7 and 14 day sacrifice times, respectively. Thereafter, the increase in lavaged AM subsided to values similar to the

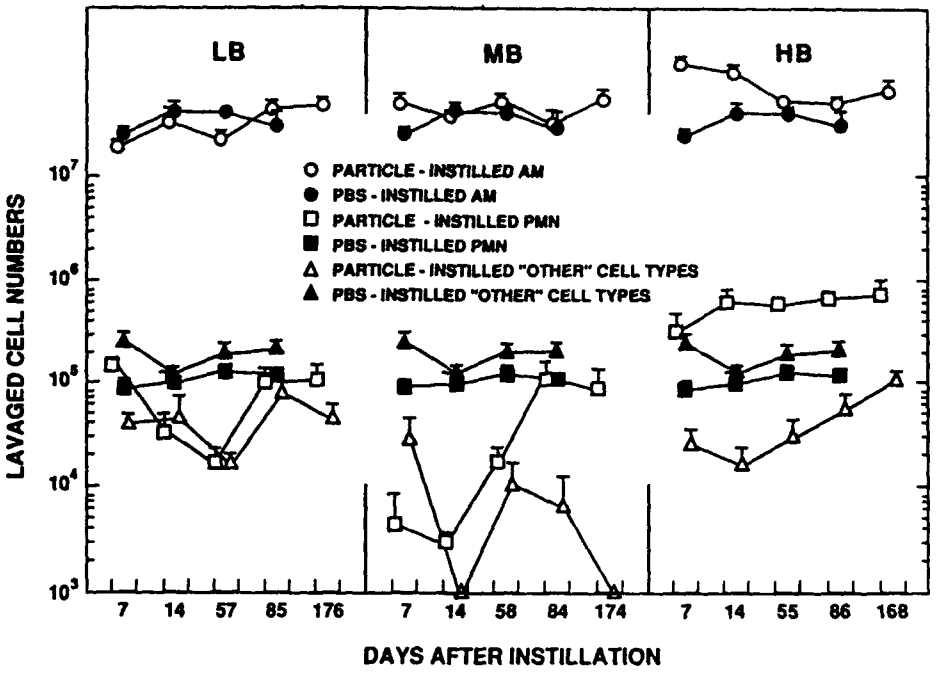

FIGURE 1
Free Cell Populations after Deposition of the LB, MB, and HB

Numbers of alveolar macrophages (AM), polymorphonuclear leukocytes (PMN) and "other" cell types, e.g., exfoliated airway epithelial cells, lymphocytes, following the instillation of PBS, the LB (A), the MB (B), or the HB (C). Daily values represent the means + S.E. of 3-8 rats per group.

numbers of AM harvested from control lungs and lungs instilled with the LB and MB of microspheres. Also illustrated in Figure 1, persistent elevations in the numbers of polymorphonuclear leukocytes (PMN) were observed following the deposition of the HB, whereas this response was not evident in the free cell populations lavaged from the lungs administered the LB and MB of particles.

Lung Retention Kinetics

The lung retention characteristics of microspheres following the deposition of the three different lung burdens, which have been described in detail elsewhere (Lehnert *et al*, 1990), are illustrated in Figures 2-4A and 4B. The coefficients and exponents of the equations that fit the retention data for the different lung burdens are summarized in Table 1. When the day 86 estimates were included in the HB data, the multiple correlation coefficients, R^2 (regression sum of squares/total sum of squares), for the LB, MB, and HB retention data were 0.98, 0.95, and 0.97, respectively. With exclusion of the day 86 data, R^2 for the HB data was slightly better (0.99).

Statistical comparisons of the slower component slopes were made by finding the reduction in the sum of squares between an unconstrained model and a model constrained to have identical slopes (Graybill, 1961). These analyses indicated that the long term slopes of the LB and MB did not differ significantly from one another, although the exponent of the MB data was somewhat lower. Hence, the deposition of up to 1 mg of the particles resulted in at most only a minor prolongation of the half-time of the longer term component beyond what was observed with the 86 µg LB of microspheres, Table 1. The longer term slope of retention of the HB (~3.7 mg), on the other hand, was significantly less than the slopes of the other two lung burdens, regardless of how we handled the day 86 data, $P < 0.05$.

Statistical analyses (Graybill, 1961) of the shorter components showed no significant differences between the slopes of the LB and MB. The shorter term component of the HB, however,

TABLE 1: Kinetics of Particle Retention of the Different Initial Lung Burdens of Microspheres

Initial Particle Burden	[No. of Particles]	A Component (Percentage of Particles)	[λ_1]*	($t_{1/2}$)**
1.6 x 10^7[a]	[8.35 x 10^6]	(52)	[0.0367]	(19)
2.0 x 10^8	[1.21 x 10^8]	(60)	[0.0323]	(21)
6.8 x 10^8[b]	[3.55 x 10^8]	(52)	[0.0197]	(35)
6.8 x 10^8[c]	[1.99 x 10^8]	(29)	[0.0185]	(37)
Initial Particle Burden	**[No. of Particles]**	**B Component (Percentage of Particles)**	**[λ_2]***	**($t_{1/2}$)****
1.6 x 10^7[a]	[7.65 x 10^6]	(48)	[0.0068]	(102)
2.0 x 10^8	[7.92 x 10^7]	(40)	[0.0058]	(120)
6.8 x 10^8[b]	[3.25 x 10^8]	(48)	[0.00013]	(5331)
6.8 x 10^8[c]	[4.81 x 10^8]	(71)	[0.0030]	(231)

*: Fraction · day^{-1}; **half-times in days.
a: The retention characteristics of the LB are closely similar to those reported for the rat following the alveolar deposition of a low burden of latex microspheres (3 μm dia.) delivered as an aerosol (Snipes *et al*, 1988).
b: Analyses performed with inclusion of the day 86 estimated lung burdens.
c: Analyses performed with exclusion of the day 86 estimated lung burdens.

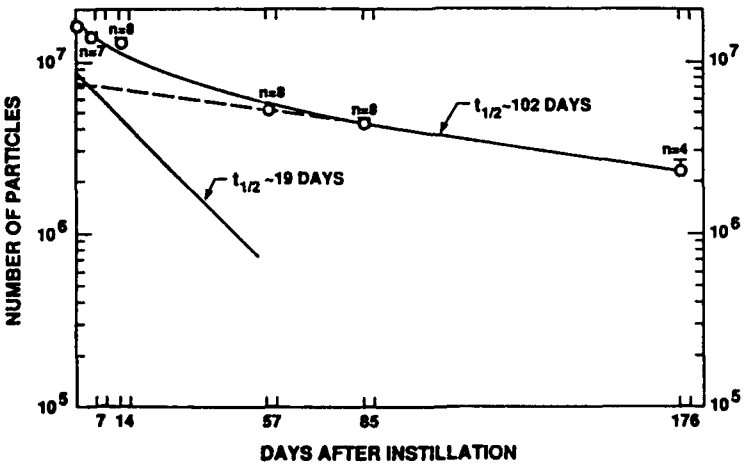

FIGURE 2
Lung Retention Kinetics of the LB of Microspheres

The more rapid and the slower term components of the retention of this lung burden are illustrated as straight lines with their associated half-times. Each value on the figure represents the mean + S.E. n= the number of animals studied per time point.

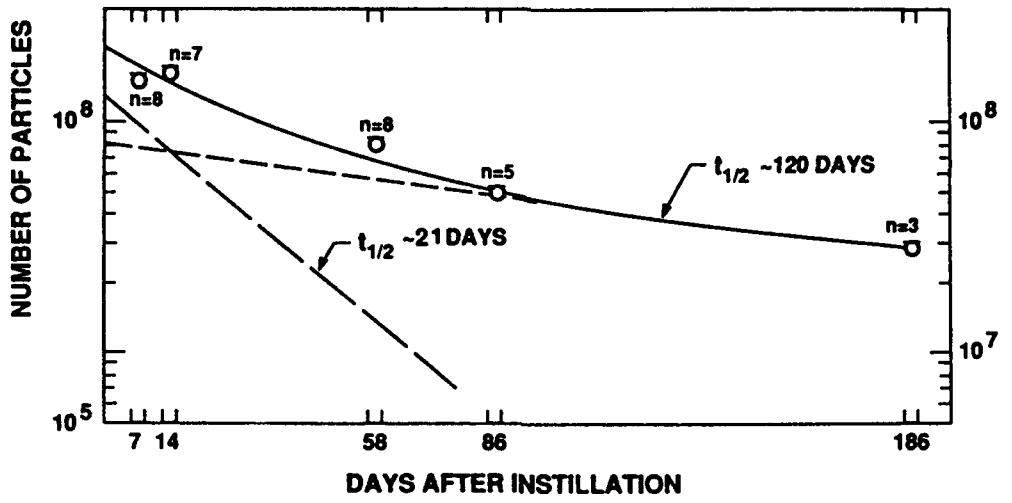

FIGURE 3
Lung Retention Kinetics of the MB of Microspheres

The more rapid and the slower term components of the retention of this lung burden are illustrated as straight lines with their associated half-times. Each value on the figure represents the mean + S.E. n= the number of animals studied per time point.

was significantly prolonged beyond the shorter term components of the other two lung burden conditions, $P< 0.05$. Overall, the half-times of the exponents for the LB and MB were similar with a value of about 20 days, whereas the half-time of the shorter term component of the HB was increased to ~35-37 days, Table 1 and Figures 2-4A and 4B.

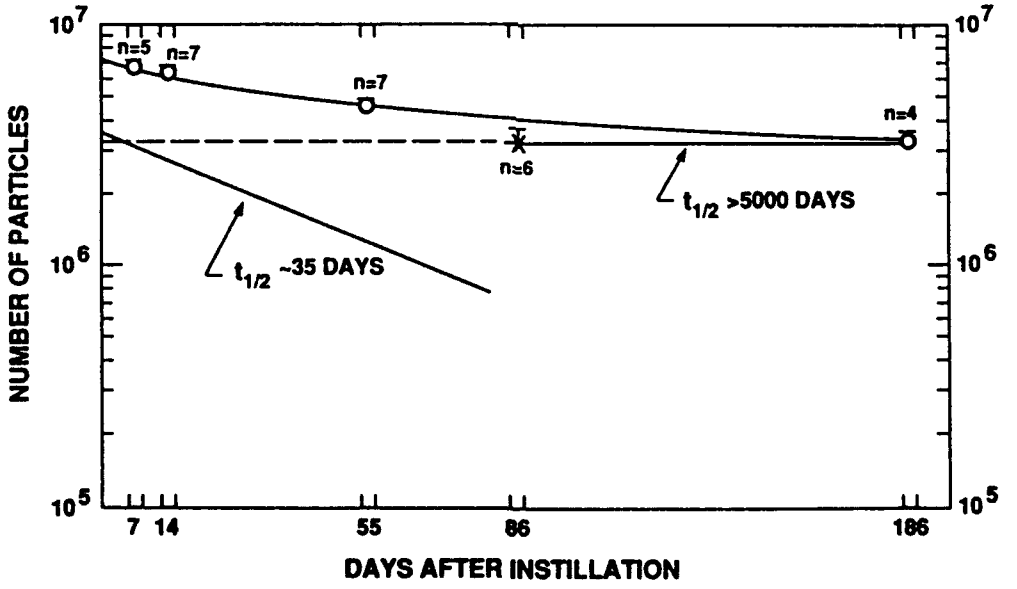

FIGURE 4A
Lung Retention Kinetics of the HB of Microspheres with Inclusion of Day 86 Lung Burden Values

The values for retained particle numbers illustrated by an X symbol at the day 86 time point represent estimated values for the retained lung burdens, as described in the Methods and Materials section. Each of the daily values represents the mean + S.E. n= the number of animals studied per time point.

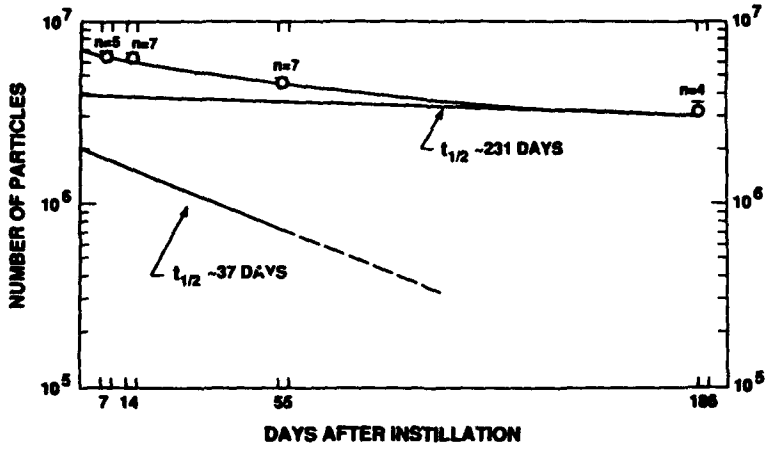

FIGURE 4B
Lung Retention Kinetics of the HB Upon Exclusion of Day 86 Estimated Lung Burden Values
Each daily value represents the mean + S.E. n= the number of animals studied per time point.

The relative percentages of the deposited particles that appeared to be removed from the lung by each component were similar for the three lung burdens when the day 86 estimated lung burden values were included in the analyses, Table 1. Upon exclusion of the day 86 values, however, the proportion of the particles associated with the shorter term component appeared to be reduced while the proportion of the particles associated with the longer term component was correspondingly increased.

Particle-Alveolar Macrophage Relationships

Particle-AM relationships during the alveolar clearance of the different lung burdens of microspheres were expressed in terms of the total estimated numbers of AM in the lung that contained a given load of particles. Semilogarithmic plots of the extrapolated numbers of AM in various particle categories at each of the sacrifice times following the deposition of the LB are shown in Figure 5. Detailed analyses of these data have been described elsewhere (Lehnert *et al*, 1989). Briefly, the apparent overall rate(s) of disappearance of AM with engulfed particles was found to increase with increasing particle burdens in the macrophages. For cellular burdens up to ~14 microspheres, the disappearance of AM from the overall AM population followed a pattern, which, like the lung retention data, could be described by a two component, negative exponential equation for each particle burden category. A plot of the later component exponents relative to AM-particle burdens showed a linear trend between the rate of later phase AM disappearance and their cellular burdens, i.e., the magnitudes of the exponents decreased linearly as the number of microspheres defining an AM category increased.

Analyses of the early components of AM disappearance also pointed to decreases in the magnitude of the exponents with increasing burdens in the AM. Unlike the lower particle burden categories, the disappearance rates for AM with ~15 or more particles were found to be best fitted with single negative exponential functions, the exponents of which appeared to be more akin to the latter component rates of the lesser particle burden categories than they were to the early component rates of AM with less than 15 particles. An outcome of the apparent differing disappearance rates of the AM from the particle categories was a gradual redistribution in the fractions of the retained lung burdens of the microspheres contained in the various particle-AM categories, Figure 6. Most prominently, only ~33% of the lung burden was in AM that contained 1-2 particles on day 7, whereas by day 176, this particle-AM category accounted for ~65% of the retained particles. We previously suggested that these findings were: 1) consistent with an enhanced rate of removal of AM via the tracheobronchial route as their cellular burdens of particles increase, and/or 2) that particles are gradually redistributed among the lung's AM population over the course of alveolar clearance concurrent with the removal of particle containing AM via the conducting airways (Lehnert *et al*, 1989b).

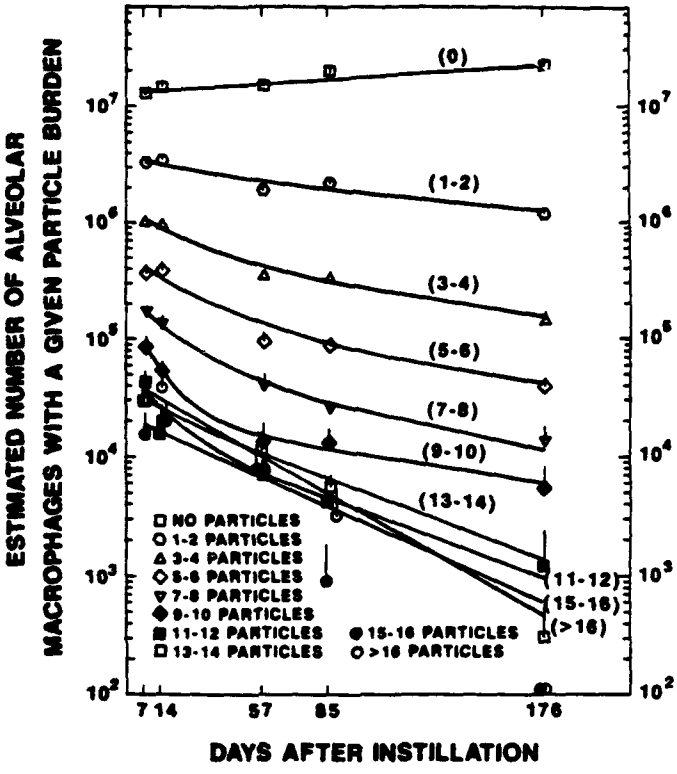

FIGURE 5
Extrapolated Numbers of All LB AM in Various Particle Burden Categories at Each Sacrifice Time

Data points represent the mean + S.E. of 4-8 lungs per time point.

Directionally similar patterns of AM disappearance from the AM population as a function of their particle loads were also observed following the instillation of the MB, Figure 7. Again, AM with the higher initial burdens of particles appeared to decrease in numbers more rapidly following the deposition of the particles than did AM with lesser particulate loads. Like with the LB, increasing fractions of the retained lung burden became progressively contained in AM with relatively lower burdens of microspheres in the cells so that no AM with high particle burdens, i.e., >50 particles per cell, were observed by day 174. Our previous postulate that particles are gradually redistributed among the AM during alveolar clearance gained further support from this component of the study in that AM in the lower particle-containing categories, e.g., 1-4 particles per cell, showed no evidence of disappearance from the total AM population over the course of the 174 day study, Figure 7. In order to determine if this latter observation was due to a failure of AM with low particle loads to translocate from the alveoli via the mucociliary apparatus, an ancillary study was undertaken in which we compared the frequency distributions of the microspheres in airway intra-luminal macrophages (AI-LM) harvested from the trachea with the frequency distribution of particles in AM harvested from the same lungs of rats at various times following the instillation of the MB. The close similarity in the particle distributions in the AI-LM and AM over a 160 day post-deposition period provided evidence inconsistent with the possibility that the previously described relative constancy in the numbers of AM containing 1-4 microspheres was due to a failure of AM with a low burden of particles to translocate from the alveolar compartment, Figure 8.

Again, as with the LB of microspheres, the net result of apparent slower rates of disappearance of AM with low particle burdens following the deposition of the MB was that increasing fractions of the retained microspheres became progressively contained in AM with relatively lower particulate burdens per cell during the clearance of the MB, Figure 9.

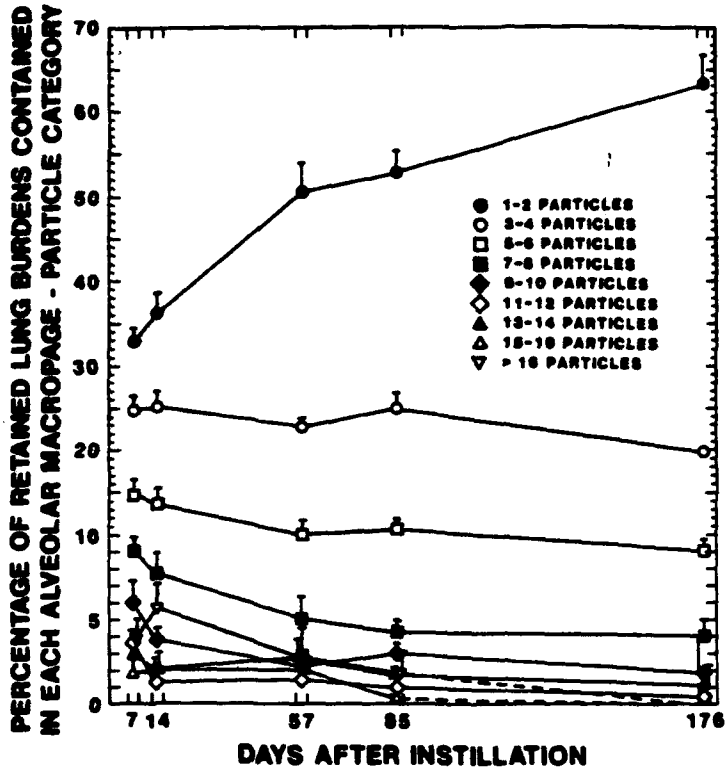

FIGURE 6
Estimated Percentages of the Retained LB of Microspheres
Contained in Each Particle-AM Category

Each of the above values represents the mean + S.E. of data obtained from 4-8 lungs.

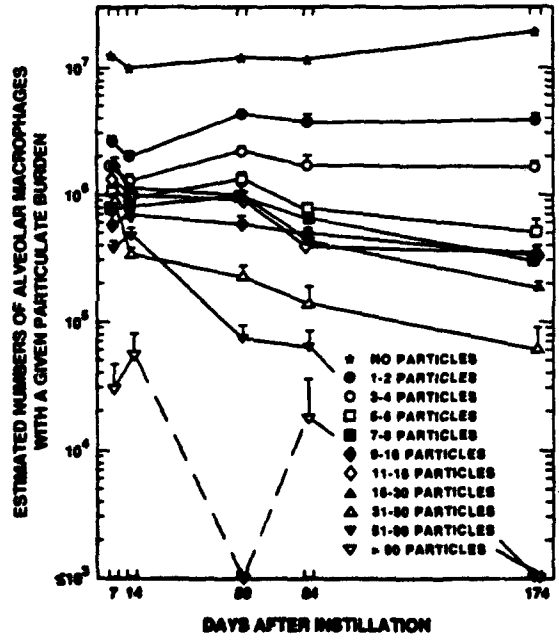

FIGURE 7
Extrapolated Numbers of All AM in Various Particle Burden Categories
Following the Deposition of the MB of Microspheres

Each of the above values represents the mean + S.E. of data obtained from 3-8 lungs.

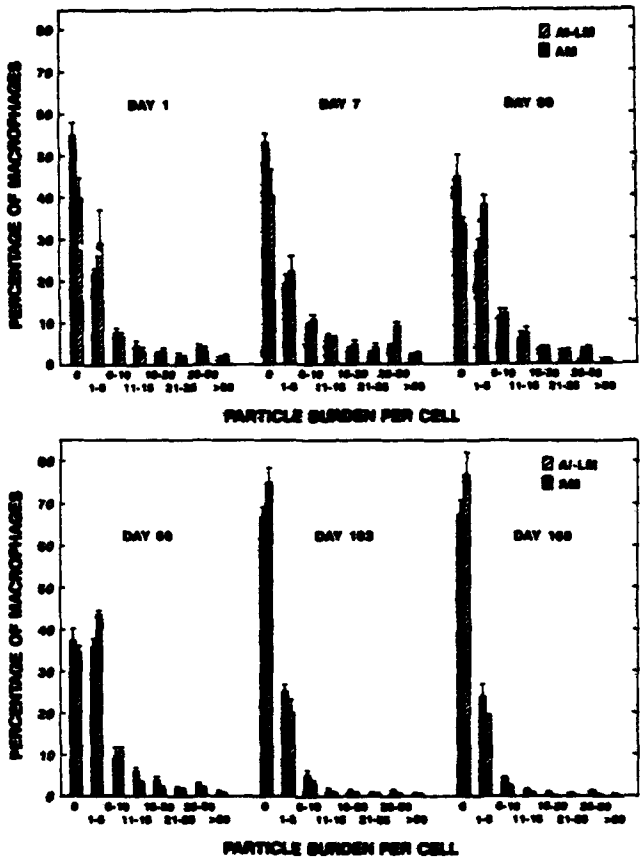

FIGURE 8
Frequency Distributions of the Particles in the AI-LM and in AM from the Same Animals on Days 1, 7, 60, 103, and 160 after the Instillation of the MB of Microspheres

Values represent the means + S.E. of data obtained from 5-6 rats per time point.

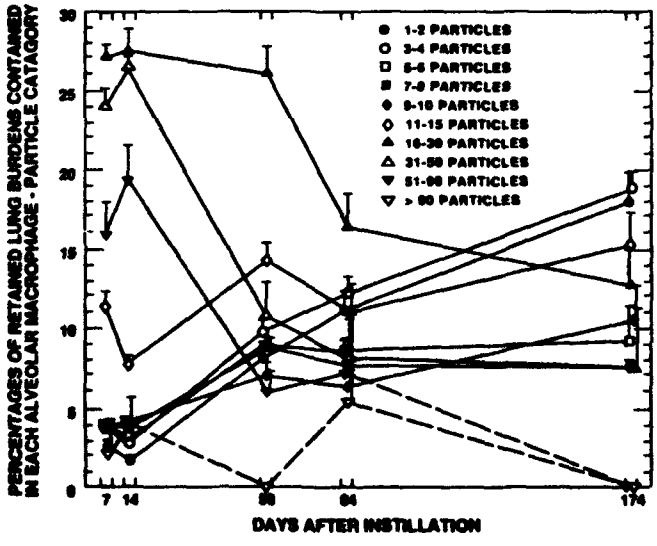

FIGURE 9
Estimated Percentages of the Retained Lung Burdens Contained in Each Particle-AM Category Following the Deposition of the MB of Microspheres

Each value represents the mean + S.E. of data obtained from 3-8 lungs per time point.

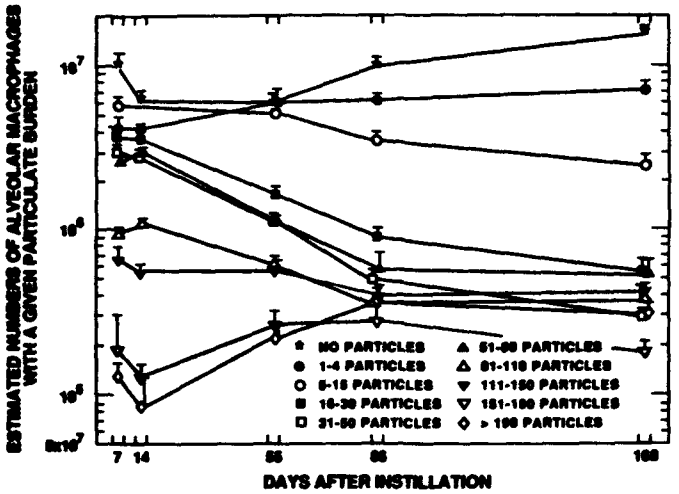

FIGURE 10
Extrapolated Numbers of All AM in Various Particle Categories
Following the Deposition of the HB of Microspheres

Day 86 values were derived from estimations of the retained lung burden at that time and a value for lavage recovery efficiency interpolated from the Day 55 and Day 168 lavage efficiency recovery data. Each value represents the mean + S.E. of data obtained from 4-7 lungs.

Particle-AM relationships during the condition of particle overload caused by the deposition of the HB are summarized in Figure 10. In this case, estimated AM numbers in the higher particle categories, i.e., 111-150, 151-190, >190 particles per AM categories, showed little overall change over the 168 day post-instillation period. On day 7 following the instillation of the HB, ~21% of the original burden was collectively contained in these particle-AM categories, and, a closely similar 23% of the original HB remained in these categories as of the last sacrifice time. Some of the AM in the highest particle-AM category were estimated to contain over 300 microspheres at each sacrifice time. Morrow (1988) has previously suggested that the removal of AM from the lung is inhibited when their mean volumetric particulate burdens are estimated to be ~60% of the normal AM volume. The relative particulate volumetric loads of the AM in these higher particle burden categories essentially equaled or exceeded the 60% value, Table 2. Reduced particle clearance of the HB was also associated with the aggregation of AM that were heavily laden with the microspheres, Figure 11. Unlike the LB and MB conditions, greater percentages of the retained HB of microspheres were progressively contained in AM in the higher particle burden categories over the course of the study, Figure 12.

Table 2: Volume Equivalents of Particles Contained in Alveolar Macrophages

No. Particles per AM	~ Volume Equivalent (μm^3)	Volume Equivalent/ Ave. AM Volume (980 μm^3)*
1 - 5	5 - 25	0.005 - 0.026
6 - 10	30 - 51	0.031 - 0.052
11 - 15	56 - 76	0.057 - 0.078
16 - 30	81 - 152	0.083 - 0.155
31 - 50	157 - 253	0.160 - 0.258
51 - 80	258 - 405	0.263 - 0.413
81 - 110	410 - 557	0.418 - 0.568
111 - 150	562 - 759	0.573 - 0.774
151 - 190	764 - 961	0.780 - 0.981
191 - 300	966 - 1518	0.986 - 1.549

*: Estimated average AM volume (Lehnert et al, 1989a).

FIGURE 11
Electron Micrograph of an Aggregate of Particle-Filled AM in an Alveolus on Day 106 Following the Deposition of the HB of Microspheres

The question arises as to whether or not the pool of heavily particle-laden AM that appeared not to translocate from the lung totally accounted for the enhanced retention of the microspheres observed with the HB. To address this issue, we re-examined the HB lung retention data after subtracting an averaged estimate of the total numbers of particles contained in the AM with 111 or more particles from each post-instillation data point. The numbers of particles subtracted was the average sum of the particles estimated to be in the 111-150, 151-190, and >190 particles per AM categories on sacrifice days 7, 14, 55, and 168; this averaged value was 1.47 x 10^8 microspheres. If the diminutions in the rates of clearance of the HB was exclusively

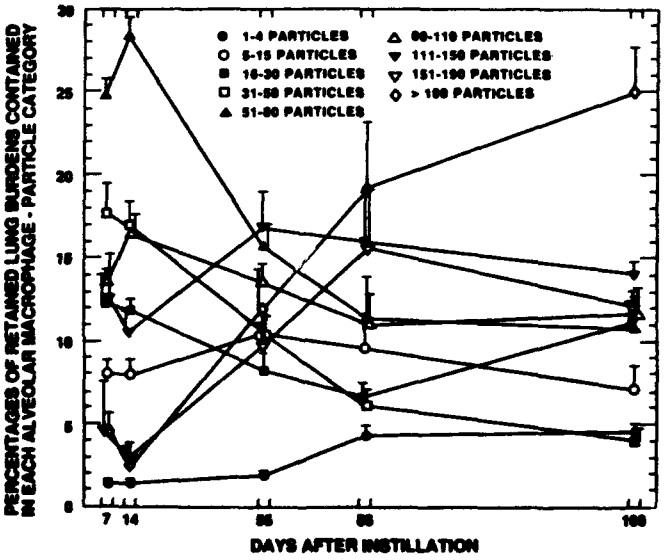

FIGURE 12
Percentages of the Retained Lung Burdens Contained in the Various Particle-AM Categories Following the Deposition of the HB of Microspheres

The Day 86 data have been reconstructed as previously indicated. Each value represents the mean + S.E. of data obtained from 4-7 lungs per time point.

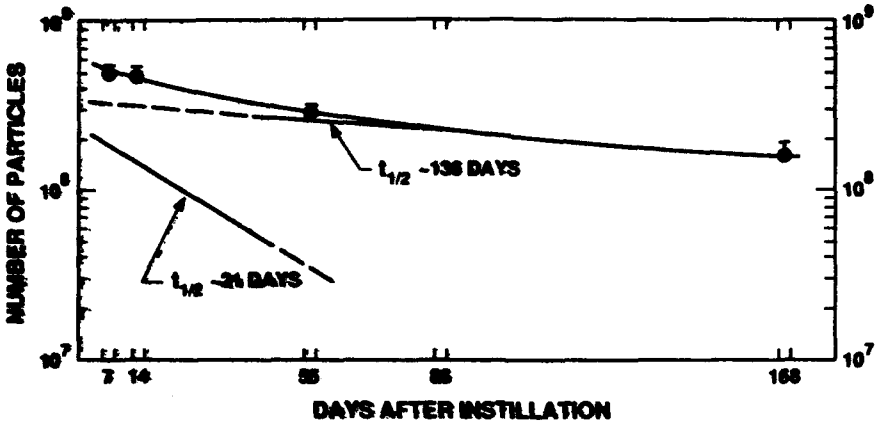

FIGURE 13
Modified Depiction of the HB Lung Retention Data from Days 7, 14, 55, and 168 Upon Exclusion of the Average Number of Particles Associated with the AM That Were Heavily Loaded with the Microspheres

The rapid and slower term components are represented by straight lines with associated $t_{1/2}$ values.

attributable to the lack of removal of these heavily loaded AM, we reasoned that removal of their collective particulate burdens from the retention data should provide a depiction of lung retention kinetics in their absence, which, in turn, should be similar to retention rates observed with the LB and MB of particles. In this exercise, the modified retention data (without inclusion of the number of particles instilled at t=0, and with or without inclusion of the interpolated day 86 retention data) were fit to the two-component model by nonlinear least squares (Draper and Smith, 1966). In doing so, the long term exponent derived from the day 55 and day 168 data, and the long term exponent derived from the day 55, reconstructed day 86, and day 168 data were held as constants as the only constraining conditions, respectively. As indicated in Figures 13 and 14, the effective elimination of the particles contained in AM that did not appear to be removed from the lung following the deposition of the HB resulted in lung retention rates that were closely similar to those ascertained from the LB and MB retention data, Figures 2 and 3. Thus, we conclude from these analyses that diminution in the rate(s) of clearance of the HB observed with

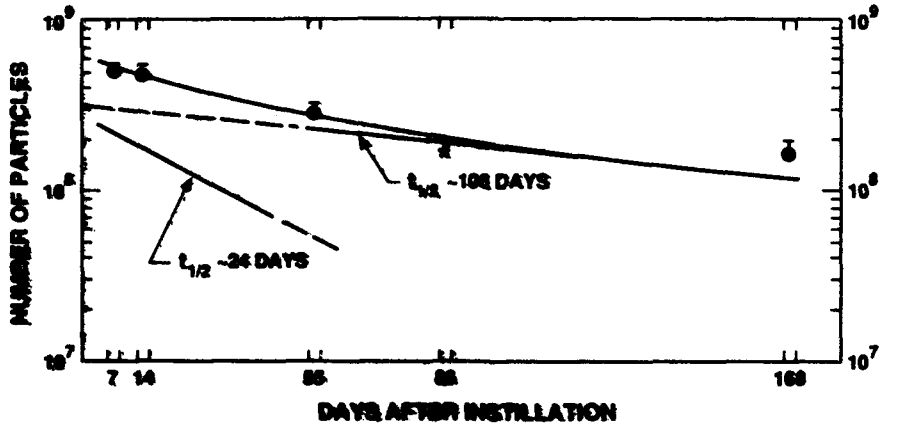

FIGURE 14
Modified Depiction of the HB Lung Retention Data with Inclusion of the Reconstructed Day 86 Data and Exclusion of the Average Number of Particles Associated with the AM That Were Heavily Loaded with the Microspheres

The rapid and slower term components are represented by straight lines with associated $t_{1/2}$ values.

FIGURE 15
Micrograph of Particle-Containing PMN and Blood Monocyte-, Pulmonary Interstitial Macrophage-Like Cells, and More Typical AM Lavaged on Day 86 Following the Instillation of the HB of Microspheres

Small closed arrow: PMN engorged with microspheres; small open arrow: particle-containing blood monocyte-, interstitial macrophage-like cell; large closed arrow: particle-laden AM.

the original retention data can be virtually totally attributed to AM containing particles at volumetric loads equal to or exceeding ~60% of their normal volumes.

Consistent with a gradual redistribution of particles among the lung's free cells after deposition of the HB, the estimated numbers of AM in the lowest particle burden category, i.e., AM containing 1-4 particles, nearly doubled over the course of the 168 post-particle depositional period, Figure 10. Additionally, particle-containing polymorphonuclear leukocytes (PMN) and blood monocyte-, pulmonary interstitial macrophage-like cells (the latter of which were scored as AM) gradually became increasingly important, yet still relatively minor, cellular reservoirs of particle containment, Figure 15. This response was not observed with the LB and MB conditions. Following the deposition of the HB and the subsidence of the acute recruitment of PMN into the alveoli in response to the microspheres (Lehnert *et al*, 1985), which is essentially complete as of ~3-4 days after their instillation, the numbers of lavaged PMN remained persistently increased above control values or PMN numbers lavaged from the LB and MB lungs, Figure 1. Moreover, the percentages of the lavaged PMN that contained the microspheres generally increased over the day 7 - day 168 study period after the deposition of the HB, Figure 16, and the cellular loads of the microspheres in the particle-containing PMN also generally increased over this period, Figure 17. PMN are widely recognized as being relatively short-lived cells. Thus, the appearance of increased numbers of these cells with their progressively increasing particle burdens implies a continual source of available free particles in the lung, such as the gradual release of particles from particle-containing AM (Heppleston, 1961; Heppleston and Young, 1974).

Validity of Estimated Total AM Numbers in a Given Particle-Burden Category

Inherent to the estimated values of the numbers of AM in various particle categories following the deposition of the LB, MB, and HB are the assumptions that *all* of the retained particles were associated with lung free phagocytes and that the particulate burdens in lavaged cells were indicative of lung free cells that remained after the lavage procedure. Some lines of evidence indicate that the first assumption is practically, but not completely, valid. While the particle size used in this study are readily phagocytized by AM and they have a relatively low likelihood of translocating across the alveolar epithelium (Adamson and Bowden, 1981), we did find that some of the deposited particles gradually translocated to the regional tracheobronchial lymph nodes, Figure 18, so that by the end of the study the lymph nodes from the LB, MB, and HB groups contained ~0.02%, 0.07%, 0.7% of the originally instilled burdens, respectively. In the rat, the majority of microspheres of the size used in this study appear to translocate from the lung to the

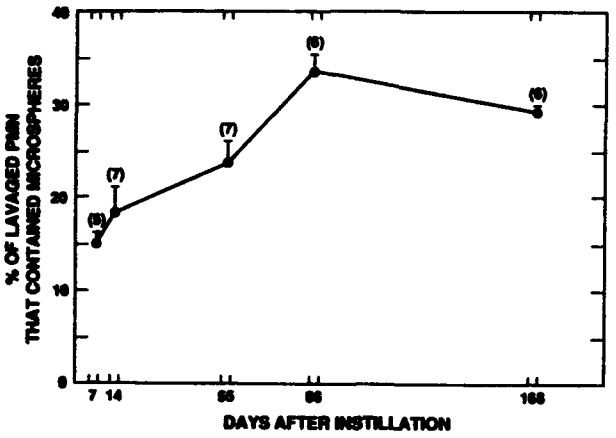

FIGURE 16
Percentages of the Lavaged PMN that Contained Microspheres Following the Intratracheal Instillation of the HB of Microspheres

Each value represents the mean + S.E. of data obtained with 4-7 lavaged lung free cell populations per time point.

tracheobronchial lymph nodes as "free" particles (Lehnert *et al*, 1986). Accordingly, the lymph nodal data shown in Figure 18 suggest that some low number of free particles, and, hence, extra-macrophagic particles, were available for passage to the regional nodes over the course of the study. The low numbers of extra-macrophagic particles presumably involved in this process, however, does not seem to constitute a major departure from the assumption that the retained particles were contained in the lung's free cell population. Another line of evidence indicating

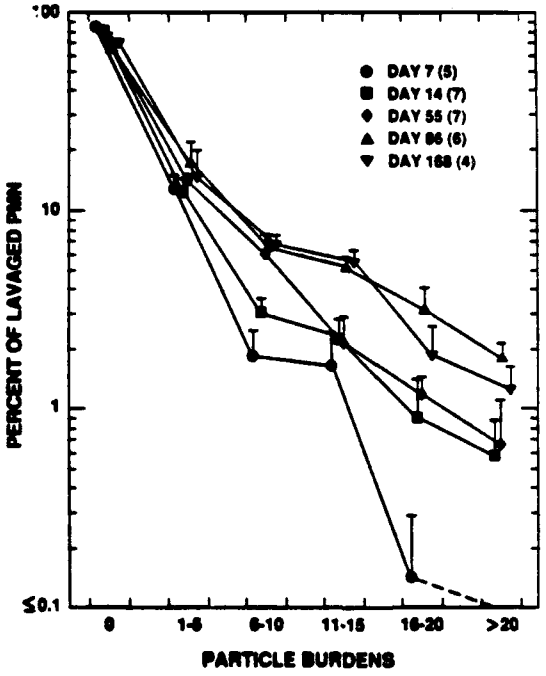

FIGURE 17
Frequency Distributions of Microspheres in Lavaged PMN Following the Deposition of the HB of Microspheres

Each value is the mean + S.E. of the indicated number of lung free cell populations studied.

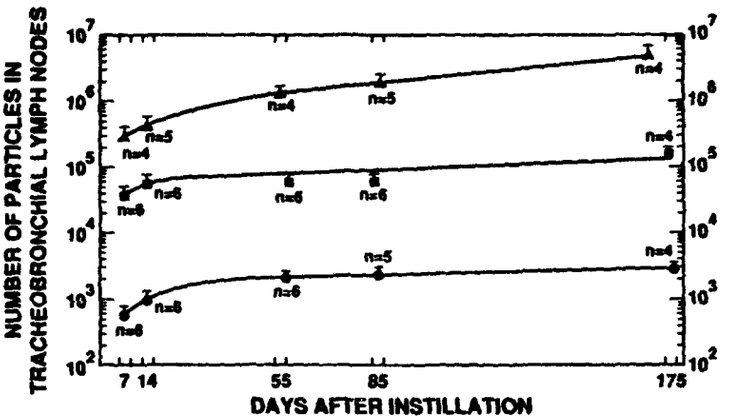

FIGURE 18
Total Numbers of Particles in the Tracheobronchial Lymph Nodes Following the Intrapulmonary Deposition of the LB (circles), MB (squares), and HB of Microspheres (triangles)

Each value represents the mean + S.E. of data obtained from 4-8 animals.

that all of the retained particles were not AM-associated comes from electron microscopic studies of lungs that received the HB. These assessments have indicated that some of the retained particles were contained in Type I cells and in the lung's interstitium in macrophages (micrographs not shown). Such phenomena have been almost exclusively observed in alveoli that contained aggregates of particle-laden AM. The relative abundance of particles in these extra-AM sites was not estimated in our study. However, assessments of the lavage recovery efficiencies (LRE), i.e., the percentages of the retained lung burdens that were recovered by lavage, indirectly provide some insight into this matter, Figure 19. Whereas the LRE were rather uniform over the various sacrifice times following the deposition of the LB and MB, some evidence was obtained that indicated that the LRE progressively decreased at later times after the deposition of the HB. Yet,

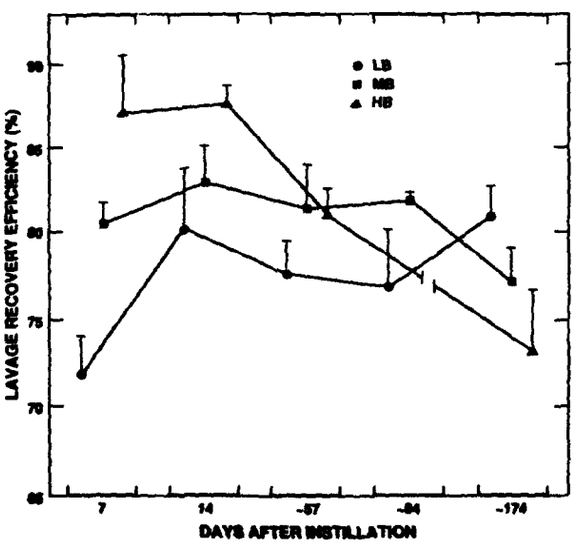

FIGURE 19
Lavage Recovery Efficiencies at the Various Sacrifice Times Following the Deposition of the LB, MB, and HB of Microspheres

Each value represents the mean + S.E. of data obtained with 3-8 lungs.

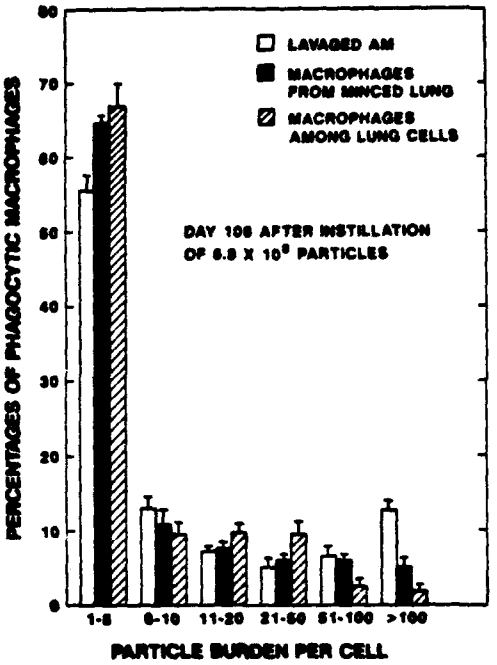

FIGURE 20
Frequency Distributions of the Microspheres in Lavaged AM, in AM From Minced Lung, and in AM From Enzymatically Dissociated Lung Tissue

Illustrated values represent the means + S.E. of data obtained with 5 individual lungs.

even if such declines were due to the containment of particles in compartments that were not accessible by lavage, potential errors introduced in AM number estimates in the various particle categories would be expected to be no greater than 10% or so. An error of this magnitude would not substantially impact on our depictions of AM-particle relationships during the clearance of the HB.

In order to seek information on the validity of the second assumption that the lavage procedure harvested AM that are representative of their unlavaged counterparts, another ancillary study was performed (Lehnert et al,1990). In this study, the lungs of rats were lavaged on day 106 following the instillation of the HB. The lung tissue was then minced and agitated to obtain unlavaged residual AM (Dethloff and Lehnert, 1987). Thereafter, single cell suspensions were prepared from the minced tissue fragments by using collagenase and mechanical agitation (Lehnert *et al*, 1985b; Dethloff and Lehnert, 1988) to harvest the AM that remained in the lung tissue following the prior two procedures. The frequency distributions of the microspheres in cells morphologically identified as AM were determined with cells obtained by the three sequential approaches. A concordance in the frequency distributions of particles in the three populations would imply that the lavage procedure is unbiased in the sampling of AM in terms of their particulate burdens. As summarized in Figure 20, the distributions of particles in AM harvested by bronchoalveolar lavage were closely similar to the particle distributions found in AM obtained from minced lung tissue and in AM from enzymatically-dissociated lung tissue.

DISCUSSION

It is generally well recognized that AM readily phagocytize a wide variety of materials shortly after the particulate materials deposit in the alveolar region of the lung. In the case of relatively insoluble, noncytoxic particles, it is also recognized that the AM with their internalized burdens of particles gain access to and become coupled to the mucociliary apparatus for subsequent transport from the lung via the conducting airways. Little detailed information is currently

available, however, regarding the actual mechanisms involved in the process of AM-mediated particle clearance from the lung or about AM-associated mechanisms that may be involved in promoting the retention of deposited particles. In this study, we have investigated particle-AM relationships during the alveolar phase clearance of low to relatively high burdens of particles as one approach to experimentally address these issues.

Of relevance to AM-related mechanisms involved during the clearance of insoluble particles generally, various lines of evidence obtained in our study have indicated that particles are gradually redistributed over time among members of the lung's AM population concurrent with the removal of AM from the lung. Although we have not identified the specific cellular bases for the particle redistributing phenomenon, potential mechanisms could include: 1) the *in situ* division of particle-containing AM and allocations of the parent AMs' particles to their progeny, 2) the *in situ* autolysis of particle-containing AM and the uptake of particles by other AM with preexisting or no particle burdens, 3) the exocytosis of particles by AM and the uptake of particles by other AM, 4) the phagocytosis of effete particle-containing AM by other AM, and/or, perhaps, 5) the direct transfer of particles from one AM to another. Experimental support for at least some of these processes comes from a variety of investigations involving AM and other endocytic cell types (e.g., Heppleston and Young, 1974; Evans *et al*, 1986; Aronson, 1963; Sandusky *et al*, 1977; Heppleston, 1961; Riley and Dean, 1978). Regardless, the impact that the particle redistribution phenomenon has on the retention of particles in the lungs remains problematic. Assuming, as our analyses of particle burdens in AI-LM and AM have suggested, that the removal of AM from the lung via the conducting airways is independent of their cellular burdens (with the exception of AM overloaded with particles), and if the alveolar removal of AM is otherwise stochastic and is a first order process, the particle redistribution phenomenon may have virtually no influence on the kinetics of particle retention. For example, the same number of particles in AM would be expected to be removed from the lung per unit of time whether 25% of the cells in the total AM population contained 10 particles per cell or 50% of the AM population contained only 5 particles per cell. On the other hand, if the particle redistibution phenomenon is predominantly due to a process(es) that involve the release of free particles from cells that have previously engulfed them, as some of our findings suggest, and if the free particles gain access into extra-AM compartments, i.e., Type I pneumocytes and the interstitial compartment, the retention of particles at these sites could be prolonged. Additionally, a redistribution of particles among the lung's AM population may also have a role in the developmental course of the particle overload condition due to high lung burdens of particles accumulated during chronic exposures to aerosols of relatively insoluble materials (e.g., Lee *et al*, 1986; Wolff *et al*, 1987; Bellmann *et al*, 1989). In this case, it is possible that the effect could be a gradual, directionally even-loading of the lung's AM with particles during the time of exposure along with continued further loading upon each bout of aerosol deposition. An outcome of this process would be that the lung's AM would tend to approach a condition of particle overload concurrently.

Another observation obtained from our analyses of particle-AM relationships during a condition of particle overload was what appeared to be the failure of AM to translocate from the lung when their particulate volumetric loads were equal to or exceeded the equivalent of ~60% of normal AM volume. As with the findings of Oberdorster *et al* (1988), which demonstrated that particles with a volumetric size equivalent to ~60% of normal AM volume were phagocytized but not removed from the lung over a prolonged period after deposition, our results as well support Morrow's (1988) original hypothesis regarding the relationship of the apparent critical volumetric load of particles in AM and their failure to translocate from the alveoli. Moreover, our findings have indicated that the numbers of AM with particulate volumetric loads equal to or exceeding ~60% of the normal volume of AM in a particle overload condition remained relatively stable over the course of our study. Thus, these results suggest that the functionally expressed particle-sequestration compartment formed by AM heavily laden with particles is virtually irreversibly preserved once formed. Relationships between the particle redistribution phenomenon and the apparent stability of the AM particle-sequestration compartment, accordingly, remain an area of puzzlement. Nevertheless, the essentially irreversible nature of a particle overload condition has also been observed by other investigators (Bellmann *et al*, 1989).

Lastly, other notable observations made in our study that may relate to the emergence of lung diseases such as pulmonary fibrosis and lung cancer in particle overload conditions obtained with a variety of different particulate agents (e.g., Lee *et al*, 1986; Mauderly *et al*, 1987; Holland *et al*, 1985, Lee *et al*, 1988) are the qualitative and quantitiative differences in the free cell populations observed during the clearance of the LB, MB, and HB of microspheres. Only deposition of the HB resulted in substantial increases in AM numbers, and the appearance of abnormally elevated numbers of PMN and blood monocyte-, pulmonary interstitial macrophage-

like cells in the lavageable free cell population at times well after subsidence of the initial free cell response to the microspheres (Lehnert et al,1985a). The role(s) these cells may play in the pathogenesis of lung diseases in the particle overload condition obviously requires further experiment study.

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Cellular Responses and Translocation of Particles Following Deposition in the Lung

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ABSTRACT

Instillation of carbon into mouse lung results in a rapid increase in cells recovered by bronchoalveolar lavage. Initially the increase is due to polymorphonuclear leukocytes (PMN), then after 12 hours, alveolar macrophage (AM) numbers increase and reach a maximum at 2-3 days. Whereas the initial increase in AM is due to migration of monocyte-derived cells, after 1 day AM numbers are maintained by proliferation and migration of interstitial cell precursors. In a particle overload situation, the number of AM recovered at 1 day peaked with a 1.0 mg dose and did not increase as the dose was raised though the duration of the maximal response was extended. At high levels, translocation of particles into lung parenchyma was seen and carbon was found in Type 1 epithelial cells, in interstitial macrophages (IM) and in hilar lymph nodes. An alveolar overload situation was induced by reducing phagocytosis and clearance. We instilled carbon to the lungs of mice depleted of leukocytes by whole body irradiation. The usual eflux of PMN and AM was delayed and reduced, leading to greater particle transfer to the interstitium and lymph nodes than after carbon alone. When silica was injected to irradiated mice, the increase in PMN and AM was reduced, and many particles reached the IM. At 16 weeks radiated mice that received silica had a higher weight of retained particles in the lungs, and collagen measurements were much higher than after silica or irradiation alone. The results suggest that alveolar overload greatly enhances particle translocation to the interstitium where secretion of any macrophage-derived factors is more likely to be effective in fibroblast stimulation.

INTRODUCTION

Pulmonary defenses are largely dependent upon the ability of clearance mechanisms to respond effectively to increased particulate loads, either in an emergency situation such as massive inhalation of microorganisms or in prolonged exposure to various industrial dusts. Large particles are filtered in the nasal passages or trapped on the tracheo-bronchial mucosa; those less than 3 μm in diameter may reach the air sacs, where they are engulfed by alveolar macrophages (AM). Large loads of dust promptly stimulate an outpouring of polymorphonuclear

Key Words: Alveolar macrophages: polymorphonuclear leukocytes: alveolar epithelium: carbon: silica: interstitial macrophages: fibrosis.

leukocytes (PMN) and new AM by a process which is thought to be the result of chemotactic factors released by alveolar macrophages following phagocytosis (Dauber and Daniele, 1980; Adamson and Bowden, 1982). The surge of PMNs, though brisk, is quickly over. The initiation and continued production of AM appears to involve factors that trigger release and division of cells in the bone marrow and within the pulmonary interstitium (Adamson and Bowden, 1981, 1982). In earlier kinetic studies of normal lung and after whole body irradiation, it was found that the AM population could be maintained by proliferation of macrophages in the interstitium with subsequent cell migration to the alveoli (Bowden and Adamson, 1980). In response to particulate load, a dual origin of new AM is seen with local division being supplemented by rapid migration of monocyte-derived cells. For example after carbon instillation to mouse lungs, the initial rise in alveolar macrophages at 1 day is not accompanied by a change in mitotic activity in the lung and can be accounted for by increased output of monocytes from the marrow. In the second phase, after 1 day, macrophagic output is supplemented by local production in the pulmonary interstitium. Futhermore, it has been shown that incubation of normal AM with carbon in vitro releases a factor that stimulates division of pulmonary interstitial cells when injected intratracheally to normal mice (Adamson and Bowden, 1981).

This system of local production of phagocytes in the lung may be regarded as an effective backup mechanism to deal with unusually heavy particulate loads, or when production of monocytes in the marrow is defective. The present studies examine the cellular responses in the lung and the translocation of deposited particles in these two situations of particle overload: a) when increasingly heavy doses of carbon particles are instilled to mouse lung, and b) when alveolar overload is induced by keeping the particle load constant but reducing phagocytosis and clearance. This is done by eliminating the inflammatory response by irradiating mice 2 days prior to instilling particles. The pulmonary reaction to carbon and silica has been compared using this model.

MATERIALS AND METHODS

In the experiments described below, 25g male Swiss Webster albino mice (CD1 strain) were used in groups of 4. All particulate suspensions were instilled intratracheally in 0.1 ml saline while the animals were under mild anaesthesia; animals receiving saline alone served as a control group.

a) Particulate Loading: Carbon suspensions of 8, 4, 1, and 0.1 mg were instilled intratracheally to groups of mice. From the particle diameter of 0.03 μm, it was calculated that the approximate number of particles administered was 2×10^{13}/mg. Other groups received 4 mg of 1 μm diameter polystyrene latex (approximately 2×10^{9} particles/mg) or 4 mg of 0.1 μm latex (2×10^{12} particle/mg). In each experiment mice were killed by intraperitoneal injection of nembutal at 1, 2, 3, 5, 7, 10, 14, and 28 days after particle injection. Some animals were used for cytokinetic studies and these were injected with 2μCi/g tritiated thymidine (specific activity 2 Ci/mmol) 1 hr before death. A tracheotomy was performed and the lungs were washed 4 times with 1 ml of normal saline, the washings were pooled and the number of cells recovered was counted using a hemocytometer. Cytospin preparations were made of each lavage and differential counts of PMN and AM were made so that the total number for each cell type could be calculated at each day studied.

After lavage, the lungs were inflated with 2% glutaraldehyde and removed. After 30 min, a small sample was postfixed in osmic acid and prepared for electron microscopy. The remainder of the lung was postfixed in formalin before embedding in glycol methacrylate. Sections 0.75 μm thick were prepared for autoradiography using Kodak NTB2 emulsion. The percentages of labeled cells were determined at each time by counting 3000 lung cells (excluding bronchial epithelium) per animal. Identification of labeled cells in experimental and control groups was made on the autoradiographs and differential counts of pulmonary cell types were made on 300 labeled cells per animal.

Some additional lungs were fixed by vascular perfusion without lavage, to examine the location of particles and cells. Lung slices were cut and fixed for

methacrylate sectioning and electron microscopy. In addition, two hilar lymph nodes were removed from each animal and prepared for electron microscopy.

b) Carbon Instillation to Monocyte-depleted Mice: Three groups of mice were used in these experiments. Group 1 received 650 Rad whole body irradiation from a ^{60}Co source at a dose rate of 110 Rad/min. The mice were held in individual sections of a plastic box and the dose was uniform over the field size used. Group 2 received the same dose of irradiation, and 2 days later the mice were given 4 mg of colloidal carbon in 0.1 ml sterile water through an intratracheal tube. Group 3 received the same dose of carbon but no irradiation. To protect against infection after irradiation, each group was given chlortetracycline in the drinking water (2 g/L) for 2 wk before irradiation and continuously thereafter. A mortality rate of about 20% was observed in both irradiated groups.

Animals were killed at the following intervals after carbon instillation: daily for 1 wk; at Days 9, 12, 14, at Weeks 3, 4, 6, 8, 12, 16, and 20. Blood was taken directly from the heart and the leukocyte count was determined. Blood smears were prepared and a differential count was made to determine the number of monocytes present at each time. The lungs were lavaged to count PMN and AM, then the tissue was prepared for microscopy as above.

c) Silica Administration to Irradiated Mice: In a separate experiment, two groups of mice were exposed to 650 Rad whole body irradiation, then 2 days later, one of these groups received an intratracheal injection of 1 mg silica (Dowson and Dobson, South Africa) in 0.1 ml sterile water. A third group of mice was instilled with silica only, while a fourth group received no treatment and served a control. Animals were killed at intervals to 16 weeks (Adamson et al, 1989).

The evaluation of white blood cells, PMN and AM in lung lavage fluids and lung structure was carried out as detailed above. In addition, extra animals from each group were killed at 16 weeks to assess fibrosis and the silica content of the lungs. From one group of four mice, the left lungs were processed uninflated for microscopy, while the right lungs were removed and frozen immediately for the measurement of collagen by hydroxyproline assay (Woessner, 1976). From a separate set of four mice, the lungs were removed, chopped and incubated in 40 per cent KOH overnight at 80°C to digest the tissue. After cooling, the solution was centrifuged at 1500 rpm for 15 min and an insoluble residue was obtained. This was washed twice in distilled water, then 1 drop was placed on a coated grid for examination by electron microscopy. The remainder was dried and the weight of the residue was determined.

RESULTS

a) Particle Loading: Animals killed within a few hours of particle instillation showed a uniform distribution of carbon throughout the lungs. Histologic examination showed that, in mice that received 4 mg carbon, PMN were first observed at 6 hrs in perivascular and peribronchiolar locations. In non-lavaged lungs, these cells were prominent in alveoli and airways after 12 hours. Carbon was seen free and in both PMN and AM; in several areas, the bronchial surface was covered by a mixture of free particles and phagocytes. Frequently PMN and to a lesser degree, macrophage-like cells, were seen passing between bronchiolar epithelial cells. The extent of the cellular influx is illustrated by the numbers recovered by bronchoalveolar lavage (Figure 1). Normally there are no PMN in the alveoli and, after 4 mg carbon, the acute inflammatory response is illustrated. PMN numbers were maximal between 12 hr and 2 d then declined to zero by 1 week. The number of AM rose 10 fold by 1 day and was elevated over a 2 week period. Many of these cells were heavily laden with particles.

The kinetics of the AM increase was examined using autoradiographs. As described in earlier studies, increased thymidine labeling was largely confined to the interstitial cell population (Adamson and Bowden, 1980). Only a small increase in labeling of free AM was seen and this was in cells with little or no ingested carbon. From the experiment using 2 mg carbon, the number of AM was plotted against the radiographic index for interstitial cells (Figure 2). This index is the product of the total labeling percentage and the percentage of labeled cells that are interstitial. It can be seen that the initial rise in AM numbers at 1 day

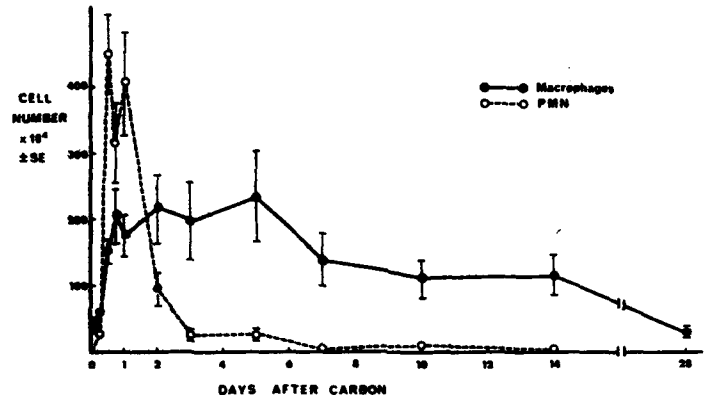

FIGURE 1. Numbers of AM and PMN in Lung Lavage after 4 mg Carbon.

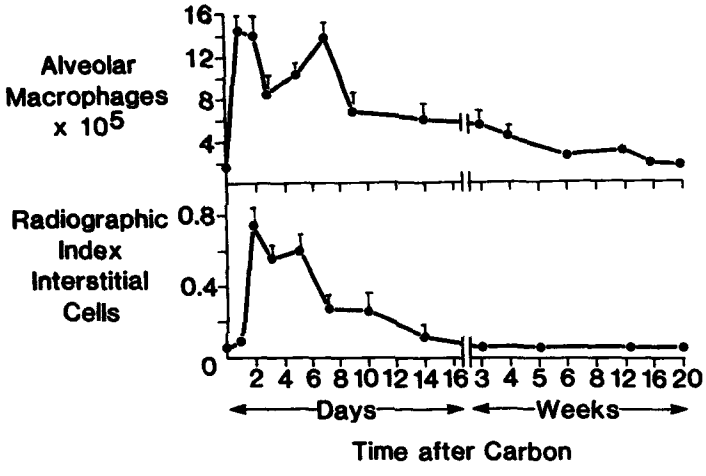

FIGURE 2. Number of AM and Labeling Index of Interstitial Cells in Lung Sections after 2 mg Carbon

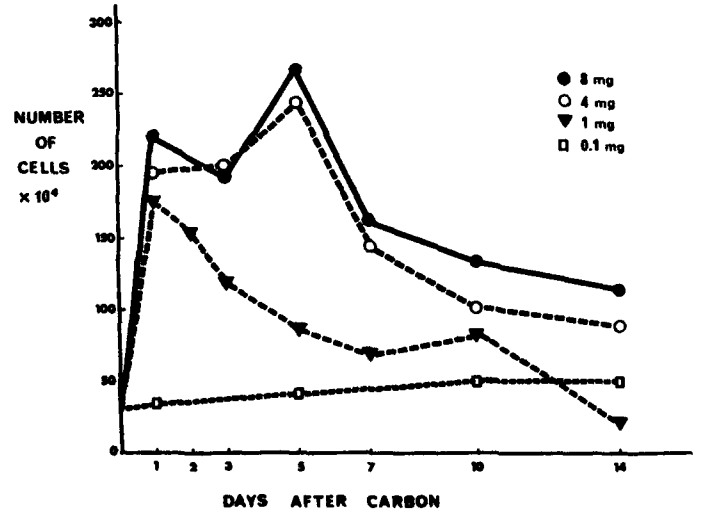

FIGURE 3. Numbers of AM in Lavage after Various Doses of Carbon

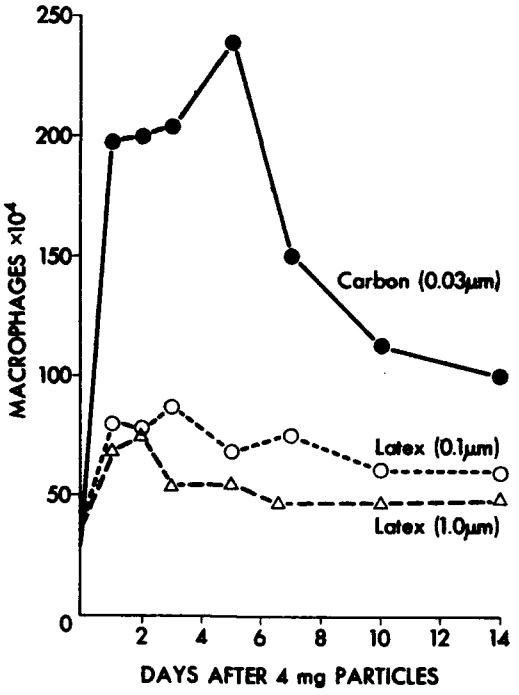

FIGURE 4. Numbers of AM in Lavage after 4 mg of Various Pariticulates

is independent of any mitotic activity in the lung, and in the interstitium in particular. However at day 2 and subsequent times, the increase in AM correlates with cell division in the interstitial cell population (Figure 2).

The effects of various doses of carbon on AM production is shown in Figure 3. The lowest dose used, 0.1 mg, produced only a small increase in AM numbers. Heavier loads up to 8 mg produced a rapid peak in cell count but there was no difference in the maximal number at the highest doses. However the duration of the maximal response was extended as the particulate load increased. In each case the cell eflux at day 1 was associated with cell migration whereas a proliferative response, predominantly in the interstitial macrophage population, was seen at day 2 and later (data not shown but similar to Figure 2). A link between number of particles instilled to the lung and AM response was seen when the cells were counted after 4 mg doses of carbon (0.03 μm diameter) and polystyrene latex (0.1 um and 1.0 μm diameter). Approximate particle numbers were calculated from diameter, suspension density and dose by weight. At the same dose by weight, most AM were recovered after carbon (approximately 10^{13} particles delivered), followed by the small latex (about 10^{12} particles) then the larger latex (about 10^{9} particles) (Figure 4).

When lung sections were examined by electron microscopy, the number of free particles seen in the alveoli increased with the number of particles administered. While most carbon was phagocytized by AM, at high levels particles were also seen in lung tissue. A few hours after injection, carbon was found in some Type 1 alveolar epithelial cells (Figure 5) and free in interstitial spaces; later particles were seen in interstitial macrophages (Figure 6). Particles were also seen in peribronchial lymphatic endothelium and in macrophages within the hilar lymph nodes. Latex particles were seen in similar locations but to a lesser extent.

b) Carbon Instillation to Irradiated Mice: All mice that received irradiation showed an immediate drop in circulating leukocytes, including monocytes, to near zero and the numbers did not begin recovery for 2 weeks (Bowden and Adamson, 1982). However these animals showed little change in AM numbers. The group of mice that received carbon 2 days after irradiation showed a doubling of AM and a steady increase over a 4 week period, however this was a much reduced level compared to

FIGURE 5. Carbon (arrows) in Type 1 Epithelium. A-Alveolus X 25,000

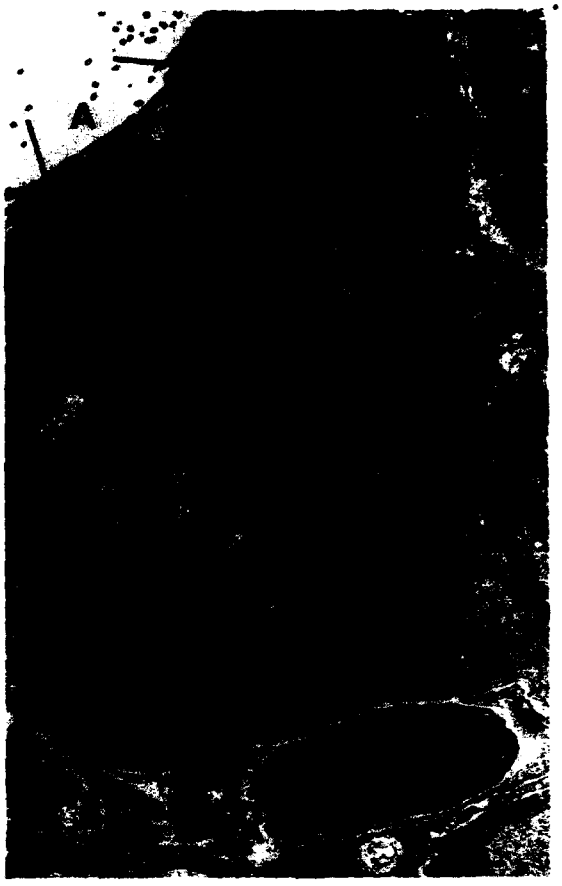

FIGURE 6. Carbon (arrows) in several Interstitial Macrophages X 7,000

the large increase seen when carbon is instilled to normal mice (Figure 7). The number of AM was still significantly higher than other groups in the carbon - irradiation group 20 weeks after administering particles. These animals were also depleted of PMN and the failure to mount an effective inflammatory response resulted in poor alveolar clearance. A large increase in particle translocation was observed with much carbon seen in Type 1 cells and interstitial macrophages (Figure 8). Particle-laden macrophages were also found in hilar lymph nodes (Figure 9). Carbon was retained in these latter locations up to 20 weeks.

c) Silica Instillation to Irradiated Mice: A similar pattern in cellular response was seen in the lavaged cell population in these experiments. The group that received silica only showed a rapid increase in cells at 12 hrs and, though the PMN number dropped, the AM increase was maintained to 8 weeks (Figure 10). When silica was given to irradiated mice, the inflammatory response was delayed and did not peak for 2 weeks. The number of phagocytes fell but was still above normal to 16 weeks. Silica particles were found in alveolar spaces up to 2 weeks and many penetrated to the interstitium. Many large interstitial granulomas were formed in the silica-irradiation group compared to a few small focal lesions in the silica-only group. In irradiated mice, silica was retained in interstitial macrophages to 16 weeks and by tissue digestion, a significantly greater residue was recovered from this group (Table). By electron microscopy, this residue was composed largely of silica particles.

Fibrosis was measured as hydroxyproline content (HYP) of the right lung at 16 weeks (Figure 11). Both silica and irradiation alone produced pulmonary fibrosis, but the combined treatment resulted in a much higher level of HYP. This

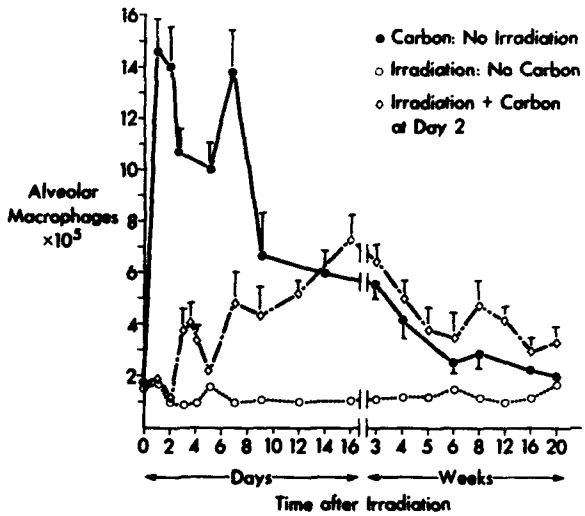

FIGURE 7. Numbers of AM Recovered after Carbon with or without prior Irradiation.

FIGURE 8. Mice Radiated before Carbon show Particles in Macrophages in Alveoli and Interstitium (arrows) X650

FIGURE 9. Hilar Lymph Node of Mouse after Carbon plus Radiation shows Macrophages with Particles X850

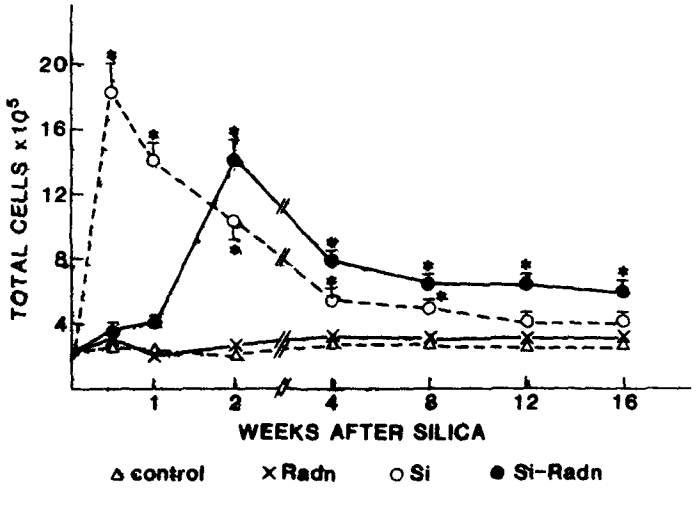

FIGURE 10. Total Cells in Lavage from Lungs after 1 mg Silica with or without prior Irradiation

value was greater than that predicted if HYP values after silica alone and irradiation alone were added together.

TABLE: WEIGHT OF RESIDUE AFTER WHOLE LUNG DIGESTION AT 16 WEEKS

GROUP (N = 4)	DRY WT (MG ± SE)
CONTROL	0.12 ± 0.04
IRRADIATION	0.17 ± 0.05
SILICA	0.40 ± 0.07 *
SILICA & IRRADIATION	0.78 ± 0.09 *,a

* EXPERIMENTAL > CONTROL, $p < 0.01$
a VALUES > ALL OTHER GROUPS, $p < 0.01$

DISCUSSION

The initial cellular response to particle instillation into the lung is granulocytic, and large numbers of PMN rapidly cross both alveolar and bronchiolar epithelium. Initially, PMN are recovered in larger numbers than macrophages and they also phagocytize and clear particles. The PMN response however is usually short lived and levels drop within 1 week after a heavy load. Even though these cells have the potential to induce injury through their enzymatic contents or free radical generation, usually an acute response, as seen after carbon, is not accompanied by lung injury. In the case of more toxic particles such as silica, the PMN response continues over several weeks, likely in response to silica-induced cell injury and retained silica in the alveoli (Adamson and Bowden, 1984).

Although the initial particle clearance involves phagocytosis by PMNs and mucociliary transport, over the longer term carbon elimination requires an adaptive increase in the number of macrophages. We have previously shown that a steady output of alveolar macrophages is maintained by a dual system whereby most cells arise by monocytic egress across the blood-air barrier and a smaller proportion are produced locally from proliferating interstitial cells (Bowden and Adamson, 1980; Adamson and Bowden, 1980). More recent evidence on the origin of the AM indicates that in normal animals, the population is maintained by local proliferation. This may occur in AM in situ (Shellito et al, 1987) or in precursor cells in the interstitium (Sorokin and Hoyt 1987), and several studies that indicate a local origin of AMs refer to pulmonary macrophage proliferation without distinguishing cellular location (Tarling and Coggle, 1982, Sawyer, 1986). In the situation of

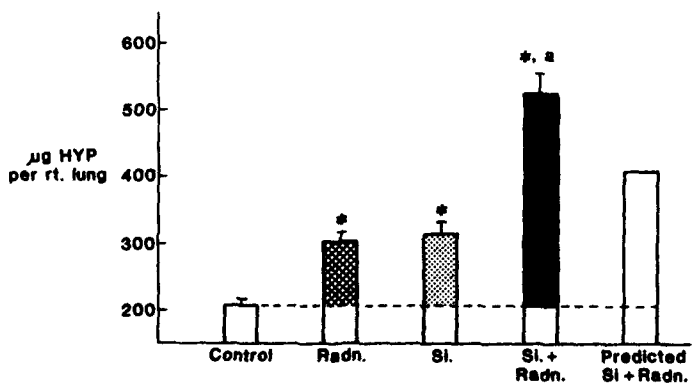

FIGURE 11. Hydroxyproline (HYP) Content of Right Lung 16 weeks after Radiation, Silica or Combined Treatment.
*, Values > control; a, value > all other groups.

particulate loading, a dual origin for the adaptive increase in AM is indicated by the biphasic cellular response seen after various doses of carbon and with polystyrene latex. In each case the number of macrophages increases sharply at 1 day with no change in DNA synthesis in the lung, indicating cellular migration whereby monocytes cross from blood to alveoli. After day 1 however, the continued macrophagic output is related to increased thymidine labeling of pulmonary interstitial cells. A small increase in labeled free AM was seen in cells that appeared immature and contained little or no ingested carbon. These may be cells of the mononuclear phagocyte series that migrated to alveoli while still capable of division.

The two components of macrophagic production respond differently to increased loading. In the case of carbon, the recruitment of new cells from monocytes appears to reach a maximum of about 2×10^6 at day 1 for all doses between 1 and 8 mg. However, the duration and level of the second phase of macrophagic production were proportional to the dose administered. The extended period of macrophagic response with increasing dose correlates with the prolonged mitotic activity in the interstitium. These results suggest that the direct monocytic route provides a rapid but limited macrophagic response to a sudden load, with cell division in the interstitium over a prolonged period providing a back up system for the generation of new macrophages.

The number of AM produced in the adaptive response is related more closely to particle number than to total dose by weight. After administering 4 mg of carbon or different sized latex particles, the overall magnitude cellular eflux was roughly proportional to the number of particles delivered, particularly when total cells recovered over a 2 week period is considered. Even at the highest dose level used, no free particles were seen in the alveoli after 1 week and, as macrophages were cleared, there was a continual reduction in carbon in the lung. However some very large carbon-laden AM were seen many weeks after particle deposition suggesting that these cells are long-lived and perhaps they may be too large, or too heavy and immobile to pass into the bronchiolar openings. Particle-laden macrophages are also seen in the interstitium at 6 months.

Not all deposited particles are cleared from the alveoli by the PMN and AM. Carbon and latex have been found crossing the cytoplasm of the Type 1 alveolar epithelial cell prior to phagocytosis by interstitial macrophages. No evidence was found for macrophage movement from alveolus to interstitium. A similar transepithelial migration has been described for other particles (Heppleston and Young, 1973; Adamson and Bowden 1981; Brody and Hill, 1982). Few if any particles have been observed in bronchiolar or Type 2 alveolar epithelium. The collection of particle laden macrophages in the peribronchiolar interstitium probably reflects particle translocation across the epithelium of neighboring alveolar wall in locations of highest particle concentration. These interstitial phagocytes may remain embedded in connective tissue for a long time or may migrate to alveoli or lymphatics. Free particles that reach the interstitial space may also be cleared directly through the lymphatic channels. A few carbon particles have been observed in the endothelial cells of bronchial lymphatic channels and may be then transported to the hilar lymph nodes directly prior to phagocytosis by macrophages. The relative importance of passage of free particles or macrophages containing particles to the lymphatics has been estimated in a quantitative study of titanium dioxide clearance which showed that, after 25 days, about 45 percent of deposited particles were eliminated by the tracheo-bronchial route while only 0.7 percent was removed by the lymphatics at low levels of exposure, and not more than 4 percent at high exposure levels (Ferin and Feldstein 1978). The present study supports the conclusion that particulate clearance by the lymphatic route is relatively small.

Deposition of high numbers of particles in the alveoli increases the chance of contact with Type 1 epithelial cells and passage into the lung tissue where particles may be phagocytized by interstitial macrophages. Carbon or latex particles do not appear to cause epithelial injury during translocation nor is there any evidence that the cellular eflux to the alveoli damages epithelial cells as no change in epithelial proliferation was seen. Particle-laden interstitial macrophages were seen in the conventional overload situation and also in phagocyte-depletion experiments where irradiated mice received carbon or silica. In each case a large increase in particle retention occurred, particularly in peribronchiolar interstitial macrophages and in hilar lymph nodes. In the case of

an inert particle such as carbon, increased retention was not accompanied by morphologic evidence of lung injury although some functional changes might accompany these peribronchiolar deposits.

The greater interaction of particles with interstitial macrophages that occurs in an alveolar overload situation may have significant consequences for lung injury. The role of macrophage secretions in fibrogenesis is well established (Goldstein and Fine, 1986), but most studies of the lung have concentrated on secretory activity of the AM (Bitterman et al 1983). The pulmonary interstitial macrophage is potentially very important because of its proximity to the fibroblast. In addition, alveolar and interstitial macrophages are different (Dethloff and Lehnert, 1988) and they may not contribute equally to the fibrogenic process. In experiments where silica was given to mice, most particles were handled by AM, some crossed to the interstitium and were associated with fibrotic regions. When silica was given to mice that could not mount an appropriate inflammatory response, many more free particles reached interstitial macrophages. In this case the amount of silica retained in the lung was greatly increased as was the fibrotic reaction, seen morphologically and biochemically. Whereas secretion of a macrophage derived factor into the alveolar space may not reach the interstitial fibroblast due to inactivation or poor passage across the epithelium, any macrophage derived factor generated in the interstitium may be directly communicated to adjacent fibroblasts (Adamson et al, 1989). During overload, the high level of particle phagocytosis by the interstitial macrophage and its subsequent activation could permit direct transfer of any growth factors to the fibroblast. Thus, it may be inferred that any pulmonary condition which results in a diminished inflammatory response, or in decreased phagocytosis, or in an excess of free particles in the alveoli, will increase the likelihood of transepithelial passage of particulates to reach the interstitial macrophage. Particle-induced changes in these macrophages and any resulting macrophage-fibroblast interaction at this location is more likely to result in structural and functional changes in the lung.

ACKNOWLEDGMENTS

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Evaluation of Alveolar Macrophage Particle Burden in Individuals Occupationally Exposed to Inorganic Dusts

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ABSTRACT

Alveolar macrophages recovered by bronchoalveolar lavage from individuals with occupational inorganic dust exposure are laden with particles. We evaluated 42 non-smoking males with long-term exposure to asbestos (27), coal (7), or silica (8), and normals (8) to determine a particle burden per 10^6 alveolar macrophages. Scanning/transmission electron microscopy and energy-dispersive x-ray analysis were utilized to evaluate the particles following bleach digestion of the cells, or of alveolar macrophage sections. There was a four-fold ($p<0.01$) increase in the number of particles in the dust-exposed. There was also a striking increase in silica particle number in the silica-exposed ($p<0.02$) but not in the other dust-exposed groups. One-third of the coal miner's cells contained silica particles predominantly <0.5 µm. In the asbestos-exposed, there was one chrysotile fiber per 35 cells, and one amosite fiber per 215 cells consistent with the known mixed exposure of workers exposed to insulation products in the United States. No crocidolite was observed in any of the cells and tremolite was identified in two controls and two workers. Computer-generated maps of elements comprising the particles demonstrated the in situ localization of the particles and identified many very small alumino-silicates, particularly in coal miners. Particle analysis is a useful technique to evaluate type and amount of exposure, to evaluate alveolar clearance, and may be useful to investigate macrophage activation.

INTRODUCTION

Alveolar macrophages protect and defend the lower respiratory tract by phagocytosing inorganic dust particles and clearing the alveolar spaces (Brain et al., 1977; Becklake, 1976; Selikoff and Lee, 1978). In this process, alveolar macrophages have been noted to be laden with dust particles in smokers and individuals with chronic occupational inorganic dust exposure (Brody and Craighead, 1975; Takemura et al., 1989; Rom et al., 1987). Bronchoalveolar lavage provides a powerful research tool to

Key Words: asbestos, silica, coal, alveolar macrophage

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sample alveolar spaces to evaluate inorganic particles and to develop a measure of particle burden per 10^6 macrophages as an in vivo estimate of exposure. Coupled with scanning transmission electron microscopy, energy-dispersive x-ray analysis, and electron diffraction, particles can be identified, sized, and enumerated. We hypothesized that these techniques would enable us to develop particle burden measurements per 10^6 alveolar macrophages, and that we would evaluate particles in situ in alveolar macrophages by scanning numerous pixels of macrophage slices with elemental analysis. In this context, we lavaged 42 non-smoking or ex-smoking for >5 year individuals with long-term occupational inorganic dust exposure to determine their particle burden and compared the results to normal volunteers without occupational exposure to inorganic dusts.

METHODS

Study Population

The study population consisted of 42 non-smoking males with chronic occupational exposure to inorganic dust (Table 1). Importantly, because cigarette smoking may result in an increased particle burden, we evaluated only those persons who were lifelong nonsmokers or had not smoked within 5 years of evaluation. The mean occupational exposure to inorganic dust was ≥20 years for each of the exposure groups and all of the chest x-rays ≥1/0 according to the 1980 ILO International Classification of the Radiographs of the Pneumoconioses. An asbestos exposure index was calculated by multiplying an index of job intensity of dust exposure times the years at the job (summed across all of the individuals' employment history). Job intensity was graded as: 4 = asbestos insulator, 3 = boiler maker, 2 = sheet metal worker, 1 = bystander asbestos exposure. Bronchoalveolar lavage and handling and analysis of the cells obtained was carried out as described by Saltini et al (Saltini et al., 1984). Eight non-smoking normal individuals without occupational exposure to inorganic dust with normal chest x-rays and pulmonary function tests served as controls.

Evaluation of Particles following Bleach Digestion of BAL Cells

One million alveolar macrophages were evaluated by optical electron microscopy for total particle number, asbestos fibers including chrysotile, amosite, tremolite, and crocidolite, silica, iron, and asbestos bodies. The cells were dissolved in 5 % sodium hypochlorite for 24 hours. Following stirring, the bleach solution was collected on an 0.45 micron pore size Millipore filter. The filter was washed with distilled water to remove excess salts, and allowed to dry. Randomly selected portions of the filter were cut out and placed, mineral side down on carbon/formvar-coated nickel electron microscope grids. The grid/membrane assembly was placed on acetone impregnated urethane foam, and capillary action drew the acetone up, dissolving the filter and leaving mineral particles on the grid. The grid was then overcoated with carbon. The grid was scanned in the electron microscope, and approximately 50 sequentially encountered mineral fibers or 100-200 particles counted, and identified by morphology, electron diffraction and energy dispersive x-ray spectroscopy (termed energy optical analysis). The counts were then converted to fibers or particles/million macrophages by a formula that takes into account size of the Millipore filter, grid square size, and number of grids scanned (Churg, 1982).

Evaluation of Particles In Situ in Alveolar Macrophage Sections

For scanning transmission electron microscopy, 10^6 alveolar macrophages were centrifuged into a pellet, fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and then dehydrated

Table 1

Clinical, Radiographic, and Physiologic Characteristics of the Study Population[1]

Group	Clinical Data: Number	Age (yr)	Exposure (yr)	Chest x-ray: (n) category[2]		Pulmonary Function Tests[3]: VC	FEV_1	FEV_1%	TLC	D_LCO	Lavage Cell Differential[4]: AM	L	N	E
Asbestos-Exposed	27	62±2	32±2	7	1/0	83±3	88±4	113±2	87±3	88±4	77±3	20±3	3±1	<1
				15	1/1									
				1	1/2									
				1	2/1									
				3	2/3									
Silica-Exposed	8	46±4	20±4	2	1/0	86±6	81±7	102±5	89±6	108±9	73±5	25±5	2±0	<1
				4	1/1									
				1	2/1									
				1	2/2									
Coal-Exposed	7	63±2	38±3	4	1/1	82±5	82±4	110±5	82±3	70±7	90±2	8±2	2±1	<1
				1	2/2									
				1	3/2									
				1	3/3									
Normal	8	39±5	0	8	0/0	91±5	95±5	105±3	94±5	97±5	83±2	15±2	2±2	<1

[1] All values presented as the mean ± standard error of the mean.

[2] Profusion of irregular opacities according to the 1980 ILO International Classification of the Radiographs of the Pneumoconioses.

[3] All values are expressed as percentage of predicted. VC = Vital Capacity, TLC = Total Lung Capacity, D_LCO = Diffusing Capacity corrected for volumes and hemoglobin, FEV_1 = Forced Expired volume in 1s; FEV_1% = forced expired volume in 1 sec/forced VC (Fulmer et al., 1977).

[4] AM = Alveolar Macrophages, L = Lymphocytes, N = Neutrophils, E = Eosinophils.

with ethanol. The cells were embedded in epon (Polybed 812) and the polymerized block was ultramicrotomed to give sections of thickness about 70 nm. Sections were left unstained and were picked up on copper grids covered with a thin formvar support film. The grids were carbon-coated in an evaporator to stabilize the support film under electron bombardment.

Analytical electron microscopy was performed in a Hitachi H700H transmission electron microscope with a STEM (scanning transmission electron microscopy) accessory. Energy-dispersive x-ray spectra were recorded using a Tracor Northern Microtrace detector and a Tracor Northern TN5500 multichannel analyzer computer system. The probe current was 1.0 nA, the beam energy 100 keV, and the spectral recording time 30 seconds. Twelve macrophages per individual and three individuals were evaluated in each dust-exposed group and normals. A STEM micrograph was recorded from each cell in order to size the particles that were to be analyzed. All visible particles (>0.03 μm) contained within a given macrophage section were probed. They were characterized according to their x-ray spectrum in the energy range 0 to 10 keV into the following groups: silica (high silicon but no other elements); asbestos (magnesium, silicon and iron); aluminosilicates (aluminum, silicon, and sometimes potassium and iron); hemosiderin deposits (large iron peak); coal (some sulfur but no other x-ray peaks). Some spectra contained weak osmium peaks due to the fixative and some chlorine due to the epon embedding medium. All spectra also displayed a copper peak originating from indirect x-ray excitation of the copper grid. These features were therefore ignored in the analyses. A small fraction of the particles (about 2%) could not be attributed to the five categories above. These particles were often calcium-rich or contained titanium but were neglected in this study. Particle sizes (defined by the largest dimension) were categorized in the ranges: <0.1 μm, 0.1-0.2 μm, 0.2-0.5 μm, 0.5-1.0 μm, >1.0 μm.

In addition to point analyses of particles within the macrophages elemental maps were also obtained from representative cells in each exposure group. A computer controlled data acquisition system based on a Digital Equipment Corporation PDP 11/60 computer with a satellite LSI 11/23 processor was used (Fiori et al., 1988; Gorlen et al., 1984; Leapman and Ornberg, 1988). The electron probe was raster scanned over a 128x128 pixel array with a dwell time of 100 ms or a 256x256 array with a dwell time of 50 ms per pixel. Spectral data at each pixel were converted into intensity values proportional to the quantities of elements that were localized at that point in the sample. Up to four elements can be mapped concurrently together with a corresponding digitized STEM image to show the morphology. The computer acquisition system can also produce electron energy loss spectroscopic (EELS) maps using a Gatan model 607 spectrophotometer (Leapman and Ornberg, 1988). This technique was used to record maps to confirm that the major constituent of the suspected coal particles was carbon.

An estimation of the volume of a macrophage from analysis of a typical section was 1% based on the formula: slice of volume ÷ cell volume = π [(diameter ÷ 2)^2 (slice thickness)] ÷ 4/3 π (diameter ÷ 2)^3 and assuming that the cross section of the cell was through its center, that the cell was spherical, the the slice thickness was 0.06 μm, and that the cell diameter was 10 μm.

Statistics

All statistics are presented as mean ± standard error of the mean. Comparison of groups was done with the Wilcoxon rank sum test. A p value of 0.05 was chosen as the level of significance.

RESULTS

Evaluation of Particle Burden per 10^6 Cells using Bleach Digestion

Following bleach digestion and scanning transmission electron microscopy with energy-optical analysis of 10^6 alveolar macrophages from

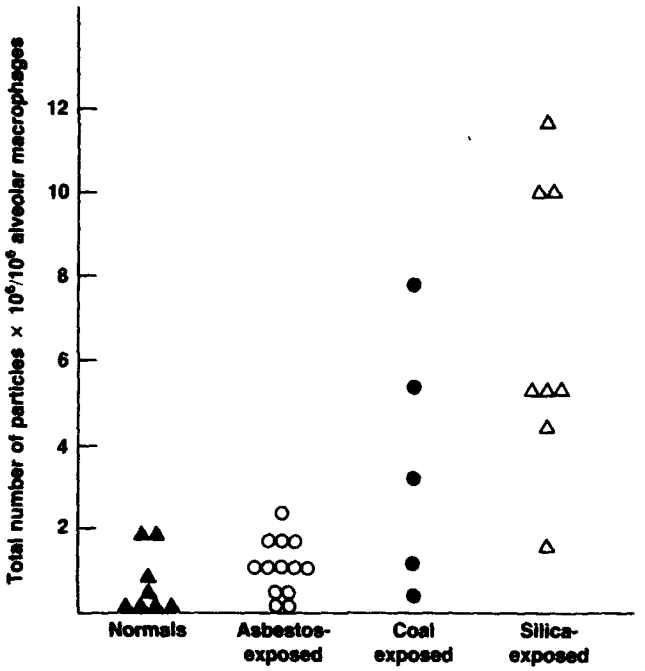


Figure 1. Total number of particles per 10^6 alveolar macrophages in normal (n=8), asbestos-exposed (n=15), coal-exposed (n=5), and silica exposed (n=8), individuals utilizing analytical optical electron microscopy. There was a greater particle burden in the dust-exposed, particularly the silica ($p<0.01$) and coal-exposed ($p<0.05$) individuals. ▲ = normal, Δ = silica, o = asbestos, ● = coal-exposed.

non-smoking individuals with inorganic dust exposure, there was a four-fold increase in particle burden ($3.9\pm0.1x10^6$ particles per 10^6 alveolar macrophages in the dust-exposed versus $1.0\pm0.1x10^6$ particles per 10^6 alveolar macrophages in the normals; $p<0.02$) (Figure 1).
This increase was primarily among silica-exposed ($p<0.01$) where, on average, up to 12 particles per cell were observed. Interestingly, 4 of 8 non-smoking normals also had up to 2 inorganic dust particles per alveolar macrophage; these particles were primarily alumino-silicates <0.1 μm in diameter. The total particle counts were asbestos-exposed $2330\pm850x10^3$, coal-exposed $3692\pm1324x10^3$, silica-exposed $6938\pm1245x10^3$ and normals $928\pm319x10^3$.

The dust-exposed individuals had more silica in 10^6 alveolar macrophages than normals ($p<0.01$) and more iron ($p<0.02$) (Figure 2); however, only the silica-exposed group was significantly greater than normals (silica-exposed $2.1\pm0.3x10^6$ silica particles/10^6 alveolar macrophages versus normals $0.2\pm0.02x10^6$, $p<0.01$).
Small numbers of talc, kaolin, mullite, mica, feldspar, aluminum, titanium, biotite, and calcium were also observed in some of the dust-exposed, particularly coal miners. Also, we compared 10^6 alveolar macrophages following two washes in culture media with 10^6 alveolar macrophages in the original lavage fluid finding no significant differences in particle or fiber burden.

The asbestos-exposed individuals had a significantly greater asbestos fiber burden per 10^6 alveolar macrophages than normals (chrysotile $28,163\pm290$ fibers in asbestos-exposed versus $1,114\pm47$ fibers in normals, $p<0.001$; amosite 4658 ± 225 fibers in asbestos-exposed versus 300 ± 294 fibers in normals, $p<0.01$, Figure 3).
There was approximately one chrysotile fiber per 35 alveolar macrophages and one amosite fiber 215 alveolar macrophages using energy optical

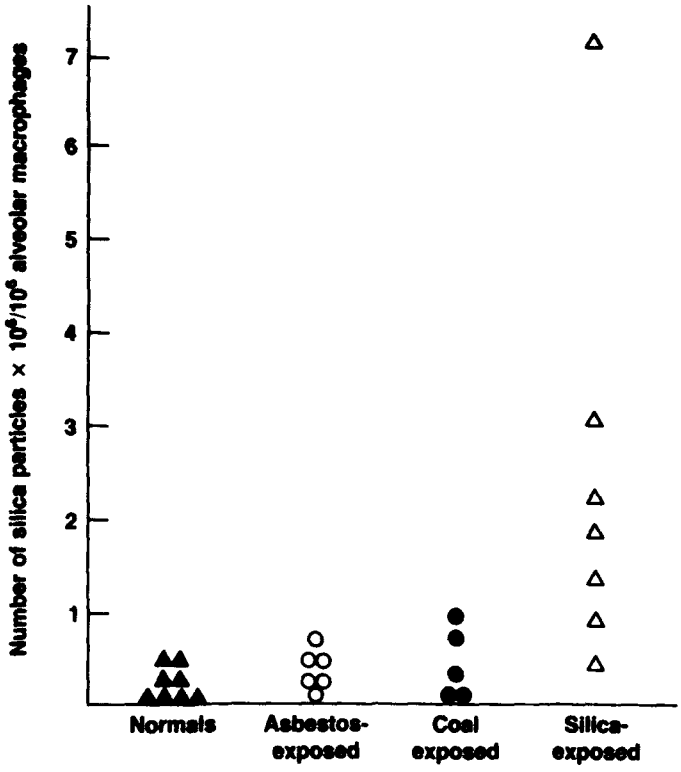


Figure 2. Number of silica particles per 10^6 alveolar macrophages using analytical optical electron microscopy. There were more silica particles in the dust-exposed ($p<0.001$) primarily due to the high numbers of silica particles in the silica-exposed individuals ($p<0.05$). ▲ = normal, Δ = silica, ○ = asbestos, ● = coal-exposed.

analysis (6-fold difference). Only two of the 25 asbestos workers evaluated had small amounts of tremolite. Four controls had small amounts of asbestos fibers; two had less than 4000 chrysotile fibers/10^6 alveolar macrophages, one had less than 2000 fibers of both amosite and tremolite, and one had less than 2000 fibers of tremolite. Similarly, the asbestos-exposed had more asbestos bodies (mean 3308±1173), $p<0.02$) than the normals where no asbestos bodies were observed. Interestingly, the mean number of asbestos bodies/10^6 cells was very similar to the mean number of amosite fibers consistent with the concept that amphibole fibers are more likely to form the core of asbestos bodies. There was a significant correlation between the number of asbestos bodies observed by optical energy analysis and the asbestos exposure index ($r=0.4$, $p<0.05$). Seven asbestos-exposed individuals also had actinolite fibers present ranging from 0.22×10^6 to 1.5×10^6 fibers/10^6 alveolar macrophages. Interestingly, no crocidolite was detected in any of the samples. Individual particle counts in the silica- or coal-exposed did not correlate with radiographic, physiologic, or lavage profiles.

Evaluation of Alveolar Macrophage Sections

In addition to analyzing the particle burden/10^6 alveolar macrophages following bleach digestion, we performed elemental analysis of all of the particles larger than 30 nm in an ultrathin 70 nm TEM section from 12 randomly selected alveolar macrophages from 3 individuals in each of the dust-exposed groups and normals. This method analyzes all of the

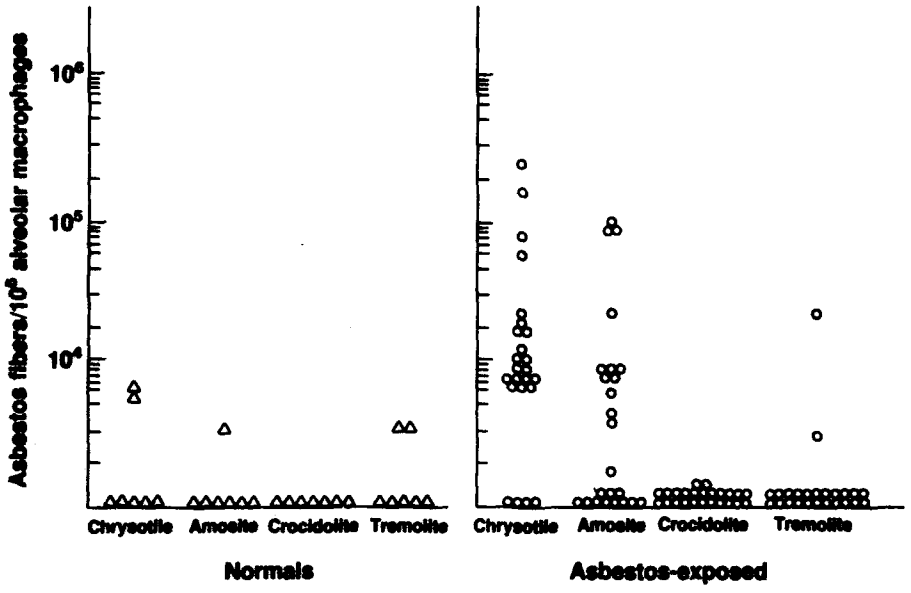


Figure 3. Number of asbestos fibers identified as chrysotile, amosite, crocidolite, or tremolite using analytical optical electron microscopy per 10^6 alveolar macrophages in normal (n=7) or asbestos-exposed (n=24) individuals. More chrysotile and amosite fibers were identified in the asbestos-exposed (both $p<0.01$). Δ = normal, o = asbestos-exposed individuals.

particles in situ in only a slice of a sphere representing approximately 1% of the total volume. The average number of particles per alveolar macrophage with this method revealed more particles compared to the whole cell bleach digestion because of the extraordinary high resolution of the technique. Two asbestos fibers in one alveolar macrophage cut in longitudinal and cross-sectional views are illustrated (Figures 4 A-D) with separate color-coded elemental analyses for silicon, iron, and magnesium. Only coal-exposed had macrophages with carbon containing particles (identified by EELS, see methods) and most of the silica and coal particles were surrounded by iron deposits (Figures 4 E-G). Asbestos fibers were observed only in the asbestos-exposed (Figure 5A). Iron and aluminum silicates were common in all alveolar macrophage sections but asbestos, silica, and coal were specific for the exposure group (Figure 5 B-E).

Silica was predominantly found in the silica-exposed and were quite small (one-third were <0.1 μm). At least one-third of the coal exposed macrophages contained silica, especially <0.5 μm in size. The coal-exposed had large numbers (mean 7.2/macrophage section and therefore approximately 720 per entire cell) of iron and potassium-containing alumino-silicates that were <1.0 μm in size (Figure 5C). The majority of the coal particles were >0.5 μm in size. Only one silica particle (<0.2 μm) was found in 36 cell slices evaluated from the normals. All of the remaining particles in the normals were predominantly <0.2 μm in size and consisted of iron or various alumino-silicates.

DISCUSSION

We utilized bronchoalveolar lavage to evaluate the particle burden/10^6 alveolar macrophages from 42 non-smoking individuals occupationally exposed to asbestos, coal, or silica with scanning/transmission electron microscopy, energy-dispersive x-ray analysis and electron diffraction finding on average of four particles

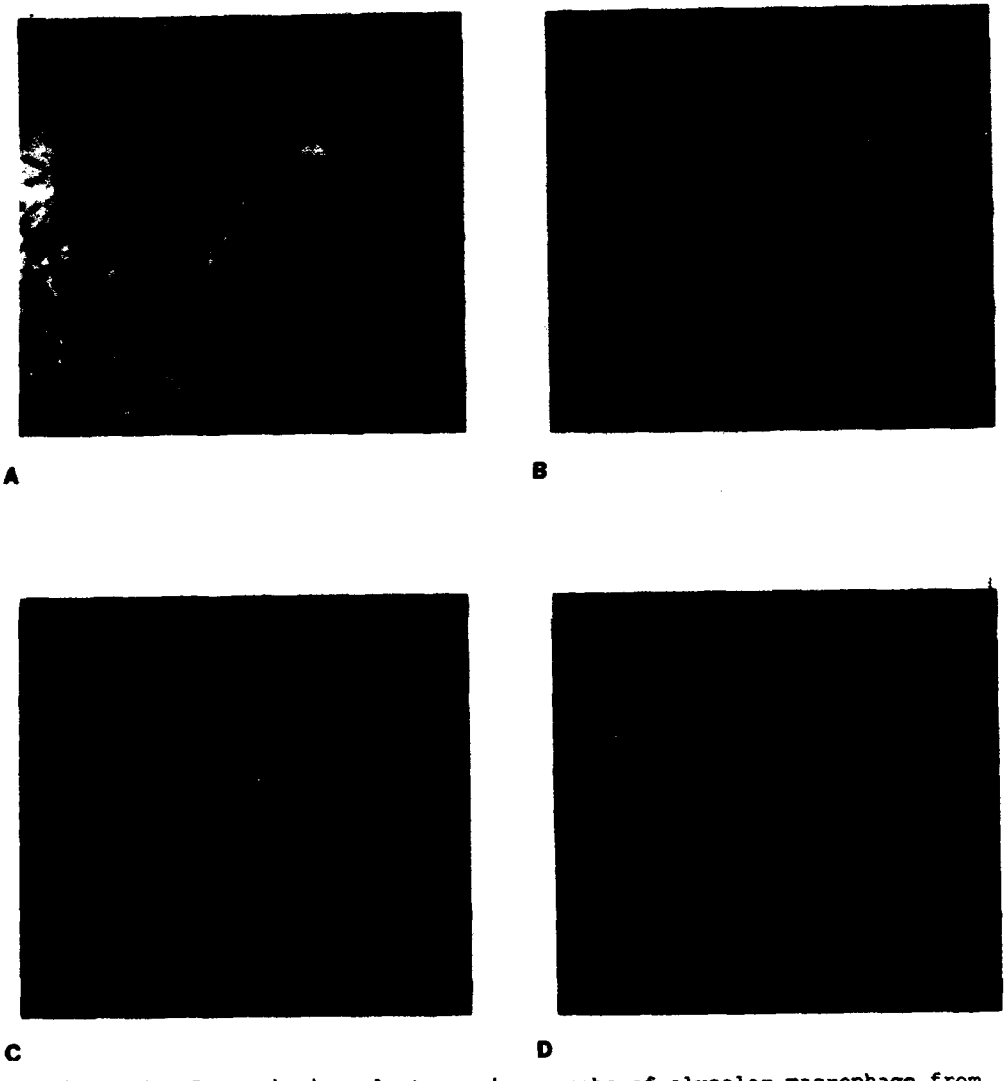
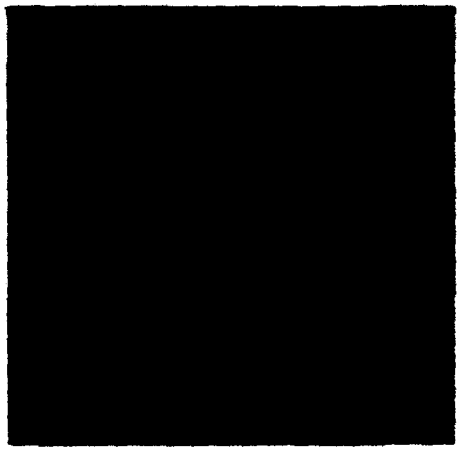


Figure 4. Transmission electron micrographs of alveolar macrophage from an asbestos-exposed individual (magnification: x3050). A. TEM section. B. Silicon (red). Elemental map of same alveolar macrophage with computer-controlled energy dispersive x-ray analysis. C. Iron (green). D. Magnesium (blue). The coincidence of silicon, iron, and magnesium in the same fiber is consistent with asbestos. One fiber is cut in cross-section and the other longitudinally.

E



F

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Figure 4. E. Transmission electron micrograph of alveolar macrophage from coal-exposed individual (magnification: x3050). F. Elemental map of same alveolar macrophage with computerized energy dispersive x-ray analysis (red = silicon, green = iron, blue = sulfur). The figure shows the typical distribution and sizes of the particles within the cells. Occurrence of iron deposits around the silica particles is observed. G. Computer map of electron energy loss spectroscopy of two sulfur-rich particles from the same alveolar macrophage identifying carbon (pink) as sulfur-containing coal particles (magnification: x6100).

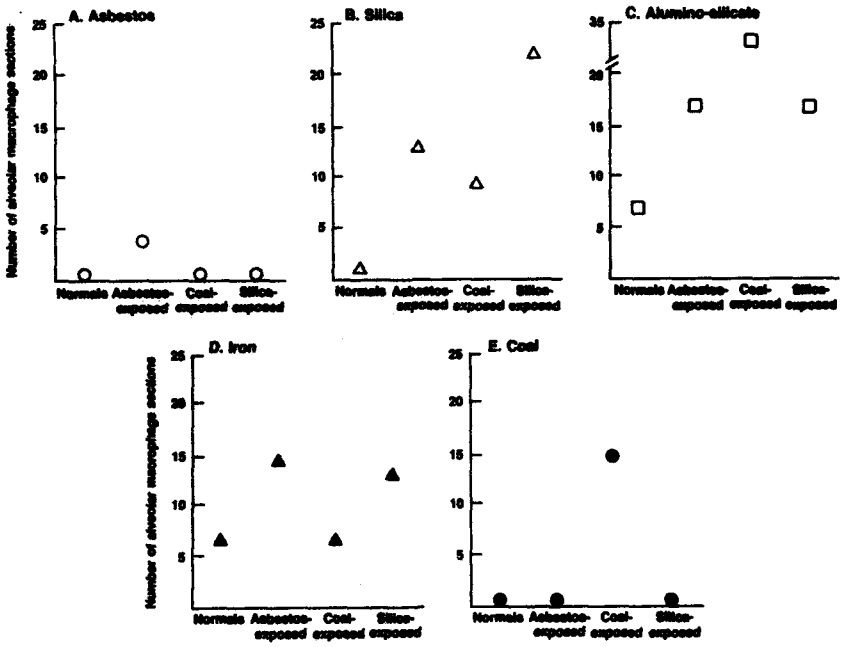

Figure 5. Number of alveolar macrophage sections containing particle types from each exposure group using scanning/transmission electron microscopy to raster-scan the macrophage section with energy-dispersive x-ray elemental analysis. There were 36 sections in each exposure group. A. Asbestos = O, B. Silica = Δ, C. Alumino-silicate = □, D. Iron = ▲, E. Coal = ●.

per alveolar macrophage compared to one per alveolar macrophage from normal individuals. Although the type of particle found reflected their respective occupational exposure, several striking observations were made. First, among the asbestos-exposed there were significant amounts of both chrysotile and amosite. Chrysotile is known to fragment and dissolve in tissue over 10-20 years (Jaurand et al., 1977), and in addition, chrysotile has been shown to be cleared more rapidly than amphibole asbestos (Churg et al., 1989a; Sebastien and Bégin, 1988). However, chrysotile is the major commercial type of asbestos constituting 93% of consumption in 1978 (Craighead and Mossman, 1982). It was also surprising to find amosite constituting 14% of the recovered asbestos fibers when it made up only 0.005% of commercial use in 1978 (Craighead and Mossman, 1982). This may reflect greater use of amosite during the period of exposure or slower clearance.

Second, particle analysis revealed many small (<0.2 μm) iron and aluminum-containing silicates in all groups but especially the coal miners. Abundance of these particles reflects the large and diverse particle exposure in underground mines, diversity in the industrial operations of foundries where most of the silica-exposed had been employed, the silicate additives to insulation materials in the asbestos-exposed, and probably urban air pollution and passive cigarette smoke exposure in the normal individuals and occupationally-exposed as well. Third, analysis of the size of the particle, albeit in two-dimensional views, revealed that approximately half of the inorganic dust particles were <0.2 μm in size, below the resolution of the light microscope. Fourth, in the coal miners' alveolar macrophages there were one-third as many silica particles compared to the silica-exposed suggesting that silica may have a role in alveolar macrophage activation in coal workers' pneumoconiosis. Several caveats are necessary, however, including the fact that we were unable to evaluate all types of

particles in all study participants' alveolar macrophages, and secondly, that we did not evaluate current smokers where the particle burden likely would have been significantly greater. In addition, since the normal controls were younger than the dust-exposed, they conceivably could have accumulated more particles if they were older from urban air pollution, environmental tobacco smoke, etc.

Previous investigations on particle analysis have primarily focused on asbestos bodies (AB) per milliliter lavage fluid returned. These studies found that the number of AB/ml correlated with duration of exposure with numbers ranging from a log mean 102.5 AB/ml in Belgian patients with radiologic evidence of asbestosis (De Vuyst et al., 1987; De Vuyst, 1982) to 79.3 AB/ml in English asbestos-exposed workers (Gellert et al., 1986). However, among 5 individuals with asbestos exposure, Johnson and colleagues (Johnson et al., 1986) listed the number of asbestos fibers per 100 macrophages finding on average 1 amosite fiber per 10 alveolar macrophages and <1 chrysotile fiber per 100 alveolar macrophages with 4 of 5 individuals having crocidolite using STEM with energy dispersive x-ray analysis. These five individuals all had a history of cigarette smoking and were exposed to asbestos in British asbestos factories or sprayed asbestos on walls and ceilings for fireproofing. Studies of French asbestos-exposed individuals reveal similar numbers of asbestos bodies and TEM-size fibers, but striking differences in fiber type with 7 of 10 individuals having >90 percent amphiboles (Jaurand et al., 1980). These differences compared to our asbestos insulators and related trades' employment likely reflect the different exposures to fiber type used in the various countries. The mean length of the fibers in the French study ranged from 2-5.6 µm and mean diameter 0.02-0.5 µm. These results were similar to size ranges from Italian investigators (2.09 µm long by 0.07 µm wide for chrysotile and 2.29 µm long by 0.15 µm wide for amphibole (Jaurand et al., 1980; Gaudichet et al., 1978; Chiappino et al., 1988). The Italian investigators also found that 16% of asbestos fibers were >5 µm in length among those with radiologic changes of asbestosis compared to 1% in those without occupational industrial exposure (Chiapinno et al., 1988). Asbestos fiber concentrations correlated with exposure histories although there were no correlations between asbestos body counts by light compared to electron microscopy. In comparing AB counts in BAL to those in tissue in 69 patients, Sebastian et al (Sebastien et al., 1988) found a highly significant correlation over 6 logs of AB numbers ($r=0.74$, $p<0.0001$). They stated that 1 AB/ml predicted a lung parenchymal concentration ranging between 1050 and 3010 AB/g which would indicate a nontrivial asbestos exposure. In addition, they estimated that BAL samples 2% of all of the AB stored in the portion of the lung lavaged. Both Sebastien et al. (Sebastien et al., 1989) and Churg et al. (Churg, 1986; Churg and Wiggs, 1986; Churg et al., 1989b) have observed an accumulation of tremolite in the lungs of Quebec asbestos miners and millers, local denizens, and in textile workers exposed to the product imported in South Carolina when the original ore sample is 99% chrysotile. We did not find a simlar increase in tremolite in the lavage cells among asbestos trades workers and would suggest that there is less tremolite in the refined end product (Churg et al., 1986b), that the chrysotile amounts in AM reflect recent exposure or differences in clearance compared to amphiboles, and that the local environment in the alveolar space is more conducive to persistence of chrysotile (e.g. the interstitial tissue inflammation may create an acidic local environment) favoring possible dissolution of chrysotile.

Evaluation of particles other than asbestos has revealed that a variety of particles may be identified including tungsten in individuals with hard metal exposure, and metals including iron, nickel, and chromium in welders and plumbers (Johnson et al., 1986). In Vermont granite shed workers, over 75% of the workers' cells contained dust compared to 5.8% in the controls (Christman et al., 1985). A silica particle burden was estimated by using the frequency distribution of x-ray energy spectrometry silicon band counts. They found that control specimens ranged from 37×10^3 to 113×10^3 counts per 100 alveolar macrophages, whereas workers specimens ranged from 100×10^3 to 981×10^3 total counts. Similarly, we identified particles of silica and

counted them per 10^6 AM finding a significant increase compared to controls. In addition, we found that the size of the silica particles were predominantly less than 1 μm.

Evaluation of mineral dust particles by scanning transmission electron microscopy with energy optical analysis in alveolar macrophages recovered by bronchoalveolar lavage has demonstrated a useful index of particle burden (De Vuyst et al., 1987; De Vuyst, 1982; Dodson et al., 1988; Lippmann, 1988). First, individuals with occupational inorganic dust exposure have a four-fold greater total particle burden compared to unexposed controls. Second, particle types reflects the occupational exposure with asbestos exposed individuals having significantly increased numbers of asbestos fibers than controls. These were predominantly chrysotile averaging 1 fiber/35 cells and amosite averaging 1 fiber/215 cells as measured after bleach digestion. Inorganic dust particles were rare in AM from unexposed controls. The particles found in unexposed controls were aluminum, calcium, and potassium-containing silicates such as kaolin or mica found in cigarette smoke and urban air pollution. Mineralogic analysis of particles in alveolar macrophages recovered by bronchoalveolar lavage provides a useful index of particle burden that correlates qualitatively and quantitatively with exposure and may provide future utility in evaluating particle-induced macrophage activation.

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Summary of Discussions from Session I: Particle-Cell Interaction—Cytology

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Speakers:

Bruce E. Lehnert, Ph.D. - *Pulmonary macrophages in a particle "overload" condition.*
Ian Y. Adamson, Ph.D. - *Cellular responses and the translocation of particles following deposition in the lung.*
W. Rom, M.D. - *Evaluation of alveolar macrophage particle burden in individuals occupationally-exposed to inorganic dusts.*

Specific questions addressed to individual speakers and general questions:

Q1. What is the relationship between phagocytosis and particle clearance from the lungs?

A1. (Lehnert) Phagocytosis, of course, is an axiomatic early component of avleolar macrophage-mediated lung clearance of many types of particles. Yet, because experimental data about how relatively insoluble particles may kinetically be removed from the lung in the absence of lung phagocytes is non existent, it remains possible that the containment of particles in phagocytes may actually prolong the retention of particles. In other words, we simply do not know how the retention kinetics of free versus phagocytized particles compare. On the other hand, it is presently reasonable to argue that unphagocytized particles have a higher likelihood of gaining access to lung interstitial sites *via* the Type I pneumocytic pathway where their retention is probably much more prolonged than when the particles are contained in macrophages in the alveolar space compartment. In the context of particle overload conditions where alveolar macrophages contain high burdens of phagocytized particles, mounting evidence is indicating that the phagocytosis of excessive particles actually prolongs the retention of particles in the lung.

Q2. Does the process of phagocytosis stimulate migration of these phagocytic cells from the lungs?

A2. (Lehnert) No clear evidence is currently available that shows a relationship between the

phagocytosis of particles and a stimulation of the migratory activities of alveolar phagocytes that may directionally favor the likelihood that the phagocytes gain access and become coupled to the mucociliary apparatus for subsequent removal from the lung. We have recently attempted to at least indirectly experimentally address this matter (Lehnert *et al.*, *Exp. Lung Res.* **16**: 451-479, 1990). In that study, we found no firm correlations between the lung retention characteristics of particles and the random and stimulated migratory activities of particle-containing alveolar macrophages *in vitro*.

Q3. Are aggregates of macrophages and particles commonly observed?

A3. (Lehnert and Gillett) Aggregates are observed after polystyrene bead instillation as well as with other particulates such as diesel soot, etc. The significance of these clustered alveolar macrophages is unknown, but in some cases they seem to persist for a prolonged period of time.

Q4. What is the rate of clearance of highly toxic particles *v s.* innocuous ones during an overload condition? Is clearance the same for highly toxic particles compared to "inert" particles of identical size?

A4. The panel agreed that highly toxic particles will adversely affect macrophage mediated particle clearance at much lower lung burdens than those needed for "innocuous" particles.

Q5. (Phipps) Are particles (beads, carbon, asbestos, etc.) ever found in interstitial fibroblasts? Evidence was shown that they are in alveolar and interstitial macrophages as well as in epithelial cells lining the lung.

A5. Lehnert and Adamson indicated that they had not observed particles in fibroblasts. Brody indicated that this was a common finding in his rat model with asbestos fibers. Phipps: it is possible that fibroblasts with particles are stimulated to proliferate and perhaps up-regulate synthesis of cytokines and collagen.

Some individuals in the audience were surprised that particles could translocate to interstitial cells. Two proposed mechanisms for particle translocation to the interstitium are (1) particle translocation through type I epithelium, and (2) phagocytosis of particles by alveolar macrophages and subsequent migration from airspace to interstitium. Clearly, there is published time-course electron microscopic evidence to support the translocation of free particles (Brody, Adamson); currently there is only indirect evidence using intratracheal instillation exposures to suggest that particle-containing macrophages migrate back from airspace to the interstitium.

Q6. Do alveolar macrophage products penetrate the epithelial barrier to affect cellular events in the interstitium?

A6. Panel: It is hard to know for certain, but it has been postulated that cytokines and other mediators may be actively transported across epithelial barriers or that they may penetrate at a more rapid rate once the epithelium is damaged. (See also A10).

Q7. What really represents an overload phenomenon?

A7. Panel and audience: An overload phenomenon depends on the particle structure, number and size of particle-types; it also depends upon the endpoints to be considered, *i.e.*,

reductions in particle clearance, inflammation, macrophage aggregates., *etc.* In addition, it has not been determined whether the critical factor in a particle overload condition is the number, mass, or surface area of deposited particles. To further complicate the issue of overload, it is possible that the association of the above stated factors may be unique for each particle-type, in particular when considering differences between particles of "nuisance" dusts and more cytotoxic particles. (See also Q4).

Q8. Why do some alveolar macrophages have an abundance of particles when others have few particles?

A8. (Lehnert) One possibility is that some alveolar macrophages are more capable of phagocytizing particles than are other macrophages. Another explanation is that the deposition of particles in the lung either as aerosol or by instillation is heterogeneous with some alveolar units receiving more or less of the particulate material. Additionally, the presence and time of appearance of an alveolar macrophage in an alveolus relative to the time when particles were deposited certainly could be expected to contribute to observations that particle distributions in alveolar macrophage populations are not uniform. For example, if a "new" mononuclear phagocyte arrives into an alveolus after particles that have deposited in that alveolus have already been phagocytized by a pre-existing resident alveolar macrophage, the new alveolar macrophage would not show evidence of particle-containment. However, as part of the particle redistribution phenomenon, this particle-free macrophage conceivably could subsequently gain a burden of the particles following the *in situ* autolysis of the older macrophage by phagocytizing particles that may be released onto the alveolar surface at times well after the original episode of particle deposition.

Panel: Experiments could be performed *in vitro* to assess the distribution of particles in freshly lavaged alveolar macrophages. If the distribution of particles within macrophages is similar to an *in vivo* distribution, then it is likely that activated macrophages phagocytize more particles. Alternatively, if all macrophages have similar numbers of particles, then the uneven distribution of particles in vivo in alveolar macrophages may reflect the focal nature of particle distribution patterns in the distal lung. Macrophages recovered from anatomic locations of high particle density would be more likely to encounter particles and hence would have a higher particle number per cell.

Additional discussions with Lehnert raised three major issues:

a. The relationship between the particle redistribution phenomenon and the apparent persistence of alveolar macrophages that are heavily loaded with particles during an overload condition is unclear. Conceivably, one component underlying the persistence of heavily loaded macrophages could be due to the sustained presence of a subset population of exceptionally long-lived alveolar macrophages.

b. That Lehnert's studies are consistent with the Morrow hypothesis that a 60% volume burden in alveolar macrophages results in overloading and depressed particle clearance.

c. That the overloading in alveolar macrophages (*i.e.*, 60% particle cell burden) is a persistent, lasting feature - *i.e.*, it does not readily resolve with time.

Q9. What factors facilitate the transport of particles through type I epithelial cells to the interstitium? Is transepithelial particle movement an active or passive process? Are epithelial cells phagocytic cells? What is the role of

epithelial injury and epithelial proliferation on particle movements to the interstitium?

A9. Three potential mechanisms discussed included (a) direct movement of particles facilitated by active uptake (cytoplasmic extensions to envelope the particles) and active transport (possibly *via* actin-containing microfilaments),
(b) epithelial injury to Type I cells and Type II cell proliferation and spreading of differentiated epithelium to replace injured Type I cells, thus incorporating the particles in the underlying basement membrane and interstitium, and finally
(c) direct transport of particles phagocytized by alveolar macrophages into the interstitium. The potential for (b) was unclear since Type I cell injury (as evidenced by cell labeling studies) and denudement of basement membranes were not evident with carbon instillation but occurred following silica instillation. Process (c) seems only to have been shown in dogs instilled with fluorescent microspheres (Harmsen, Bice and Muggenburg). Process c also appears to occur in rats (Gillett *et al.*, *J. Aer. Med.* 2, 1, 1989, 29-38).

Q10. Questions were raised regarding transport phenomenon and secretions of mediators by lung epithelial cells. Some individuals from the audience were dubious about epithelial transport and suggested that if it occurred it would be very slow. Additional questions related to the transmigration of growth factors from alveolar regions to interstitium.

A10. (Brody) Particle or fiber transport through epithelial cells is possible and likely would be rapid. Data have been published previously and demonstrates particle translocation from airspace to epithelial compartments within 1-3 hours after deposition. No data is available regarding the issue of transepithelial transport of growth factors from alveoli to interstitial regions where they could impact on target cells such as fibroblasts.

Q11. What is the mechanism of increased surfactant levels with particle overload?

A11. The enhanced secretion of surfactant may be related to the development of Type II cell hypertrophy and hyperplasia and may also affect alveolar macrophage clearance function.

Q12. The relative merits of experimental routes of exposure such as instillation *vs.* inhalation were discussed.

A12. There seemed to be a consensus that instillation of particles into the lungs of animals creates a local overload situation. Therefore, it is extremely difficult to extrapolate animal data from instillation models to humans.

Q13. The issue of pulmonary macrophage kinetics during a particle overload condition was discussed at length.

Briefly, it is difficult to reconcile the long-term presence (*i.e.*, 1-2 years) of aggregated macrophages within alveoli with current knowledge regarding the half-life of these cells (i.e., 4-6 weeks). It seems likely that aggregated macrophages turn over (perhaps by autolysis) under these conditions. Thus, it is conceivable that several generations of macrophages are involved during an overload condition. It is clear that further research is needed in this area.

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Particle-Cell Interactions: Lung Fibrogenesis

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Many inhaled particulates can cause a fibrotic response of the lung. Lung fibrosis, whether defined pathologically, biochemically, or physiologically, may occur in either an acute or chronic time frame after the fibrogenic insult. Several biochemical changes in lung collagen are associated with the later stages of this response. These include increased amounts of total lung collagen, a change in the ratio of type I to type III collagen (which may occur in response to some, but not all, fibrogenic stimuli), and changes in the relative content of hydroxylysine and of hydroxylysine-derived cross-links in fibrotic lung collagen.

Early events after initial exposure to a fibrogenic agent include acute lung injury, cell damage or cell death, lung edema, and lung inflammation. The relationship between these early events and the eventual outcome, lung fibrosis, is an area currently under very active investigation. It is also probably the component of the lung's response to injury that we least understand. A complex array of mediators and factors have been, and are being, described that may modulate the biochemical interactions and communication between cells in the lung. The possible role of such signal molecules in relating early lung damage to subsequent irreversible structural and functional alterations of the lung has not yet been defined. Many investigators assume that early and late events are linked by signal molecules that cause the selection of specific subpopulations of mesenchymal fibroblasts with enhanced proliferative activity and/or altered phenotypic expression of collagen synthesis.

INTRODUCTION

The later stages of the response of the lung to particulate insult via the airways are generally thought to be somewhat stereotyped and to a great extent independent of the chemical nature of the particles; that is, the lung shows a surprisingly limited

Key Words: Collagen/fibrosis/silica/growth factors/cytokines/animal models

range of responses to this type of challenge. Late stage responses that have been well described in animal models and in diseased human lungs include fibrosis, emphysema, tumors, granulomatous lung lesions, alveolar lipoproteinosis, and what is seemingly no response other than the presence of dust-laden macrophages. Many of the other papers presented in this symposium deal with various aspects of these potential responses of the lung. We will focus on the fibrotic response with a special emphasis on the relationship between biochemical alterations in lung collagen synthesis and pulmonary fibrosis. We will also survey current concepts of the role(s) of inflammatory cell mediators, cytokines, and various growth factors as agents that link and amplify the acute responses of the lung to injury with the late-stage responses such as fibrosis.

Collagen Synthesis in Silicosis and Other Lung Fibrosis Models

When rats are intratracheally instilled with very high doses (e.g. 25-50 mg) of crystalline silica, i.e., quartz, there are several well-characterized changes in total lung collagen metabolism. The simplest question to ask from an analytical point of view is whether there is more collagen in a silicotic than a normal lung. The answer is yes: there may be as much as four to six times the amount as found in age-matched controls (Reiser et al., 1983). There seems to be a dose-response relationship between the amount of quartz instilled and the amount of collagen (hydroxyproline) deposited in the lung (Swensson, 1971), such that it may take several months to appreciate the increased collagen content of rat lungs after instillation of relatively low doses of crystalline silica (i.e. $\leq$ 10 mg). However, at higher doses of silica such an increase in lung collagen can be appreciated quite rapidly, as early as 1-2 weeks after instillation of 50 mg in our studies (Reiser et al., 1982). In this respect, silicosis does not differ from other animal models of experimental pulmonary fibrosis. For example, intratracheally instilled bleomycin causes an increase in rat lung collagen that can be detected two to three weeks after instillation (Hesterberg et al., 1981). An important difference between silica and bleomycin, ozone, butylated hydroxytoluene and other pneumotoxicants seems to be in the continuing deposition (for at least a year after instillation) of excess collagen in lungs of animals in the silica model. The other pneumotoxic agents all seem to cause a rapid increase in lung collagen to a plateau value, which is usually 20 - 50% higher than that in age-matched controls. There are other animal models available of so-called 'progressive fibrosis', usually involving insult with two agents (e.g., bleomycin plus 70% oxygen, butylated hydroxytoluene plus 70% oxygen, bleomycin plus various immunosuppresive agents) in a carefully timed sequence. These latter models result in persistent fibrosis, with an increased content of hydroxyproline in lung (Haschek et al., 1982). However, peak values for accumulation of hydroxyproline are reached within weeks of insult, in contrast to the continuing increase over one year observed with silica.

Such observations of increased lung collagen in silicosis (and other animal models) quite naturally give rise to the question of whether the excess collagen deposited in the fibrotic lung is different from normal collagen. The answer is clearly yes, but it took a long time to find this out, and therein lies another important difference between silica and other fibrogenic agents. Our initial

experiments with silica as a fibrogenic agent were based on our naive (and, it turned out, erroneous) expectation that it might give rise to a slow, progressive fibrosis that in some respects would act as a model for chronic pulmonary fibrosis in humans (sarcoidosis or idiopathic pulmonary fibrosis [IPF]). The pioneering studies of Kang and co-workers (Seyer et al., 1976) showed that normal lung in humans contains about a 2:1 molar ratio of type I to type III collagens, and that these two types account for most (> 90%) of the total lung collagen present. These workers examined the ratios of collagen types in lungs obtained *post mortem* from patients dying of IPF and found a shift towards an increase in type I collagen, with a new ratio of about 5-6:1, on average (Seyer et al., 1976). We later demonstrated that similar shifts take place in the pool of newly synthesized collagen in animals with lungs acutely damaged by fibrogenic agents such as bleomycin, ozone, and paraquat (Reiser and Last, 1981). Therefore, it seemed logical to perform similar analyses in lungs from rats exposed to silica. To our surprise, despite the increases in collagen synthesis rate and the greatly increased collagen content of the silicotic lungs, we found (Reiser et al., 1982, 1983) that collagen was synthesized and deposited in the normal ratio of types I:III, 2:1, from one week to one year after instillation of the silica. In this respect, silicotic fibrosis is unique among the animal models of lung fibrosis we have examined (Reiser and Last 1981, Haschek et al., 1982), and it is also different from the situation in acutely (Last et al., 1983, Shoemaker et al., 1984) and chronically (Seyer et al., 1976) fibrotic human lungs. At present, we have no explanation for this difference between silica and other fibrogenic agents. We find it a fascinating reason for continuing to study silicosis, in that we would very much like to understand the underlying biochemical and cellular mechanisms whereby the silicotic lung is able to mount this unique response while rapidly laying down large amounts of collagen. The role of increased levels of type III collagen mRNA reported in lungs of animals exposed to silica by inhalation in these findings remains to be clarified (Vuorio et al., 1989).

Collagen Cross-links in Fibrotic Lung

We have also examined another important property of lung collagen, its higher-order structure as determined by the ability of collagen chains to become crosslinked to one another by lysine derived, covalent, bonds. In this process, specific lysine or hydroxylysine residues are oxidized to their corresponding aldehydes, which can then react with each other or, more importantly, with ϵ-NH_2 groups of other lysine or hydroxylysine residues (via a Schiff-base intermediate) to form difunctional (and trifunctional) crosslinks (see Figure 1). We have speculated elsewhere (Last 1985) that there are good theoretical reasons for assuming that there might be molecular markers of 'fibrotic collagen' that differ from 'normal collagen', such that the lung might modulate the ultimate metabolic fate of these collagen pools in different ways. Such speculations led us into a systematic examination of collagen crosslinking in normal rat and mouse lungs and in lungs of rodents with experimentally induced fibrosis. To do this, we exploited new techniques of HPLC on reversed-phase columns that we developed for this purpose (Reiser and Last 1986).

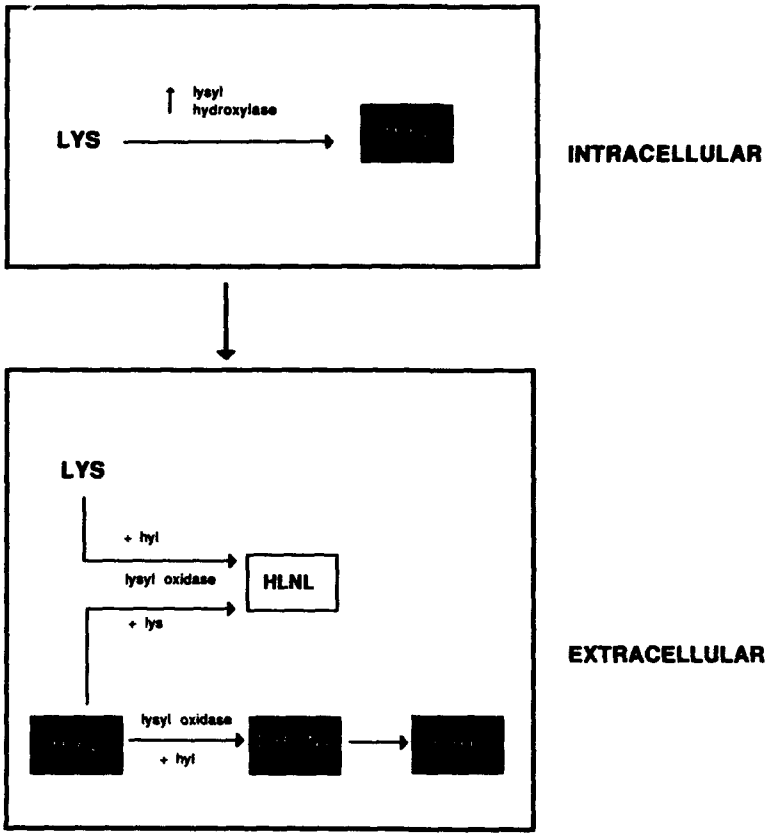

Figure 1
Collagen crosslinking in lung. Important steps are indicated. Abbreviations are defined in the text. Shaded boxes indicate key steps affected in the fibrotic lung (see text for details).

Two observations from experiments with silicotic lungs are directly relevant here. When we reduced lung tissue with NaB^3H_4 to stabilize and label the difunctional crosslinks of interest, we found the ratio of dihydroxylysinonorleucine (DHLNL) to hydroxylysinonorleucine (HLNL) in normal lung tissue to be about 3-4:1 for all samples from age-matched control rats. The DHLNL:HLNL ratio for the silicotic lungs was about 8:1 at one and four months after instillation, and about 11:1 for the six-month and nine-month samples. These changes are consistent with an increase of about 50% in the hydroxylation of lysine to hydroxylysine also observed in the collagen of these silicotic lungs (Reiser and Last, 1986). The second observation we made concerned the non-reducible ('mature') trifunctional cross-link hydroxypyridinium (OHP), derived from the condensation of three residues of hydroxylysine. Not surprisingly, OHP content of the collagen in lungs of the silicotic rats gradually increased over the duration of the experiment as compared with age-matched controls. Remarkably, at nine months after silica instillation the lungs contained about twice the normal lung content of OHP expressed on a per collagen molecule basis and substantially more than this (about 8 times the normal amount) expressed on a "per lung" basis. Thus, we identified the following changes in 'silicotic

collagen': (1) higher content of hydroxylysine, (2) greater ratio of DHLNL:HLNL, (3) higher content of OHP. The first two changes were appreciable by one month after silica instillation (the first time point we studied), while the increased content of OHP became apparent by four months after silica instillation.

We have had the opportunity to do similar studies in rats and mice receiving intratracheally instilled bleomycin, in mice receiving bleomycin plus oxygen, in lungs from infants dying of respiratory distress syndrome of the newborn, a (pre)fibrotic lung disorder (Reiser et al., 1986), and in lungs from adults suffering from various acute and chronic fibrotic lung diseases (Last et al., 1990a). Our results are consistent with those from the silica model: a rapid shift in DHLNL:HLNL ratio and a long-term increase in OHP content where animals or patients survived long enough for this change to manifest itself. In this sense, then, the silica model is completely consistent, as far as we can determine, with all other animal models of fibrosis and the changes occurring in diseased human lungs. More important, perhaps, is our observation that 'normal' and 'fibrotic' lung collagen are different, and that DHLNL:HLNL ratio or OHP content, or both, are molecular markers for 'fibrotic' collagen.

Is the excess hydroxylation of collagen (and its crosslinks) that we are observing related to increased levels of lysyl hydroxylase, the enzyme catalyzing this unique post-translational modification of collagen, in damaged lungs? We know that the activity of lysyl hydroxylase is indeed increased in many animal models of pulmonary fibrosis (Last, 1985; Last et al., 1990b). On the other hand, the extent of hydroxylation of lysine residues in collagen is also thought to be controlled by the rate of folding of collagen chains while they are being synthesized. The rate of synthesis of collagen chains may also differ among different types or clones of cells, and may be a factor in defining 'fibrotic collagen'. These kinds of questions would appear to be approachable experimentally with modern techniques of biochemistry and cell biology.

Lysine hydroxylation appears to be increased in lung collagen from fibrotic animals. The mechanisms responsible for abnormal collagen synthesis, at least at the level of lysine hydroxylation, are apparently operative in vitro. We know (i) that slices from lungs of rats given bleomycin synthesize collagen containing an increased relative content of hydroxylysine as compared with the collagen synthesized by lungs from normal rats, (ii) that the increased hydroxylation of lysine seems to occur predominantly, if not exclusively, on the alpha1(I) chain of Type I collagen, and (iii) that there is an increased activity of the enzyme lysyl hydroxylase in extracts of lungs from rats administered bleomycin (Last et al., 1990b).

Our observation that the increase in lysine hydroxylation is located almost exclusively on the alpha1(I) collagen chain is consistent with previous studies, both in vivo and in vitro. For example, studies of collagen type ratios in various models of fibrosis, as well as in human lung disease, have shown that fibrotic lungs contain increased type I collagen relative to type III collagen (Seyer et al., 1976; Reiser and Last, 1983; Last et al., 1983; Shoemaker et al., 1984). In vitro studies have shown that prefibrotic lungs preferentially synthesize Type I collagen in amounts great enough to account for most or all of the increase in collagen synthesis that can be quantified in such lungs (Reiser and Last 1981). We could envision that the prefibrotic lung contains at

least two discrete populations of cells, presumably fibroblasts, that synthesize collagen. Resident lung fibroblasts present prior to insult with bleomycin (or other fibrogenic agent), would continue to make normal lung collagen, i.e. a 2:1 ratio of Type I to Type III collagen and a normal level of lysine hydroxylation. "New" fibroblasts, i.e., those newly recruited to the lung or normal (active or quiescent) cells stimulated by growth factors or other mediators, would produce fibrotic lung collagen, i.e. predominantly or exclusively Type I collagen with a higher level of lysine hydroxylation. Thus, we could speculate that the increase in lysine hydroxylation of Type I collagen is a biochemical marker for cells synthesizing "fibrotic collagen". Increased lysyl hydroxylase activity in bleomycin-treated lungs is also consistent with the presence of this hypothetical "fibrogenic subtype" of fibroblasts, since such a subtype might well be characterized by alterations in enzymatic activity. There are precedents in the literature for the suggestion that increased hydroxylation of lysine in collagen is associated with higher levels of lysyl hydroxylase activity in the cells actively synthesizing collagen (Murad and Pinnell, 1987). On the other hand, it is also possible that increased lysine hydroxylation is a result of a decreased rate of formation of triple helical structure, as been observed in other diseases (Kirsch et al., 1981; Bateman et al., 1984; Wenstrup et al., 1986; de Vries et al., 1986).

There are several studies that support the idea that fibroblasts in (pre)fibrotic lungs are fundamentally different in some way from normal lung fibroblasts. For example, Absher et al. (1984) have found that fibroblasts from lungs of bleomycin-treated rats are larger, migrate more rapidly from cultured explants, and grow and replicate more slowly than fibroblasts from lungs of normal rats. Hildebran et al., (1987) found evidence that collagen producing cells, presumably fibroblasts from the lungs of bleomycin-exposed rats have a higher rate of production of collagen than do cells from control rat lungs. Clark and co-workers (1982) have suggested that decreased PGE_2, which is known to suppress fibroblast growth, might explain the increased proliferation of fibroblasts observed from lungs of rats administered bleomycin. Mosely et al. (1986) found that bleomycin strongly potentiated the stimulation of fibroblast proliferation induced by fibroblast growth factor or platelet-derived growth factor.

The structural implications of overhydroxylation of collagen lysine residues are not yet clear. Unlike hydroxylation of proline residues to form hydroxyproline, lysine hydroxylation is not thought to affect the stability of the collagen triple helix. It seems reasonable to speculate that an immediate sequela of overhydroxylated collagen is an alteration in collagen crosslinking. There is an increase in the dihydroxylated collagen crosslink dihydroxylysinonorleucine that is detectable within 1-2 weeks of the induction of pulmonary fibrosis (Reiser and Last, 1986; Reiser et al., 1986). Long-term changes in collagen crosslinks include an increase in the maturational product of dihydroxylysinonorleucine and hydroxylysine, the trifunctional crosslink hydroxypyridinium (Reiser and Last, 1986; Reiser et al., 1987). In addition, there are suggestions in the literature that in diseases other than pulmonary fibrosis, similar changes in collagen crosslinking occur, consistent with overhydroxylation of lysine residues (Bailey et al., 1975; Moriguchi and Fujimoto, 1979; Barnes et al., 1976; Brickley-Parsons et al., 1981; Skirving et al., 1984). In view of these observations,

it is clearly of some interest to identify the specific residues that are being overhydroxylated in the alphal(I) chain. Assuming that increases in lysine hydroxylation do not occur randomly along the chain, we would predict that lysine residues at the N- and/or C-terminal sites, which are known to participate in collagen crosslinking, might preferentially show increased hydroxylation.

There remain many unanswered questions regarding early alterations in collagen composition and synthesis in the lung destined to become fibrotic. The relative roles of various cell types in lung collagen synthesis are not well understood. Most workers assume that altered lung collagen metabolism represents a change in interstitial fibroblast number or gene expression, but all of the cells of the lung (except perhaps the pulmonary alveolar macrophage) are able to actively synthesize some types of collagen in culture. In addition, the role of mediators elaborated by inflammatory effector cells recruited to the damaged lung in modifying collagen metabolism by resident lung cells is an area of great interest and uncertainty in understanding lung fibrosis (Clark et al., 1982; Phan and Thrall, 1982). Further experiments in cell or tissue culture systems will be required to answer these questions. Such experiments will be facilitated by the identification of markers for the fibrotic phenotype in cultured lung fibroblasts, if indeed such markers exist. It remains to be proven that the increased hydroxylation of lysine in fibrotic lung collagen is such a marker for cells actively synthesizing collagen in tissues destined to become fibrotic.

The similarities and differences between silicosis and other models of experimental lung fibrosis continue to fascinate us. Key questions remain unanswered that may be further examined by such comparative studies. For example, if fibroblasts make 'fibrotic collagen', as most workers in this field assume, how are silicosis fibroblasts able to make 'fibrotic collagen' without at the same time shifting the ratio of collagen types being produced? Is the ability to make 'fibrotic collagen' a property of certain cell clones or is it under the control of factors produced by other lung cells, as suggested by several workers? Is 'fibrotic collagen' different enough from 'normal collagen' to be a rational target for therapy of these lung diseases that are at present incurable? The key role of the T lymphocyte in the pathogenesis of granulomatous lesions such as silicotic nodules is well recognized. TNF-alpha and IL-1 from macrophages probably play an important role in the development of the granulomatous lesion (Kunkel et al., 1989). IL-1 can induce IL-2 production by T cells, which in turn acts as an autocrine signal for growth and proliferation of effector T lymphocytes. TNF-alpha also seems to be important in the formation of granulomas. Various other thus far incompletely characterized cytokines seem to play an important role in granuloma formation as well.

Putative Cytokines Involved in the Development of Lung Fibrogenesis

Several growth factors and cytokines that could potentially modulate lung fibroblast activity have been described in the literature. Two recent comprehensive reviews of lung cytokines have suggested roles for PDGF, IL-1, IL-6, basic FGF, TGF-a, TGF-b, TNF-alpha, IGF-1, and many other yet to be determined mediators in the etiology of lung disease (Kelley, 1990; King et al., 1989). The

chemical properties of each mediator and its putative biological actions have been described in great detail in these two review articles. Although these analyses might lead to a better understanding of the development of lung disease and provide a descriptive basis for the possible involvement of these cytokines in lung diseases, they fail to provide specific reasons why the expression of a certain cytokine product at a certain time or in a certain anatomic location will induce or enhance the development of lung diseases such as pulmonary fibrosis. Furthermore, interpretation of such findings is complicated by the fact that several different cell types can apparently produce the same cytokines under appropriate conditions, and that the various effector cells studied can produce a battery of different cytokines and/or growth factors. In other words, there is most probably no deficiency in the production of these growth factors by the normal lung. To further complicate this concern is the fact that all of these cytokines are probably present in the uninjured lung in a quantity well over that needed to exert its biological action (based on RIA, ELISA, or bioassay measurements) on fibroblast growth; yet, an aberrant growth of interstitial cells does not normally occur. Why not? We believe that there must exist in the normal lung a homeostatic mechanism by which the activities of these cytokines, growth stimulation and inhibition, are balanced and regulated.

Based on this hypothesis, it is reasonable to assume that the normal lung is dynamically producing various growth factors and inhibitors. Under the normal, or unperturbed condition, a homeostatic balance is thereby achieved. In order to understand the pathogenesis of lung fibrosis, it will be necessary to understand the nature of this homeostatic balance among these cytokines on the regulation of the life cycle of cells of the lung; further, the control by the lung of synthesis of the various collagen types, the quantity of collagen, and even the crosslink content of collagen will also be indirectly under the control of this cytokine balance. There must exist an Ying-Yang relationship in such a balanced cytokine production, and we hypothesize that the pulmonary alveolar macrophage plays a key role in this process. Among those cytokines thus far identified in lung, we find observations consistent with this hypothesis. For example, lung macrophages secrete both TGF-a and TGF-b. TGF-b in combination with PDGF, which is also secreted by macrophages, stimulates fibroblast growth; however, TGF-b also inhibits fibroblast growth when combined with TGF-a/EGF. Furthermore, PDGF is an important competence factor for quiescent fibroblast cells. Without such a competence factor, fibroblasts would not proliferate in response to progression factors, such as fibronectin, IGF-1, and EGF. However, the effects of the presence of PDGF in tissues can be hypothetically down-regulated by an alpha 2-macroglobulin which is also secreted by the macrophage (Bonner et al, 1989; Huang, 1989). Another mechanism that may be active in the lung can reduce the activity of PDGF through an oxidation-reduction pathway. PDGF is active when both its A and/or B chains are joined together by disulfide bonds, and it is inactive in the reduced form (Paulsson et al., 1987). Therefore, despite the ubiquitous presence of a significant number of macrophages in the lung, the life cycle of the lung fibroblasts need not be affected, as any PDGF produced by quiescent macrophages may be maintained in the reduced, inactive state under homeostatic balanced conditions.

When such a putative homeostatic mechanism is directly or indirectly challenged by various environmental insults, the

TABLE 1

Cytokine and Effector Factors Produced by Various Cell Types

Cell Type	Factor
Platelet	PDGF EGF TGF-β Endothelial cell growth factor
Fibroblast	IGF-1 (synonym: somatomedin) PDGF, IL-6, IL-1
Lymphocyte	γ-IFN, TGF IL-1, IL-6, other lymphokines
Macrophage/monocyte	TGF-α, β, IGF-I IL-1, IL-6, CSF FGF, PDGF TNF-α Several poorly characterized other factors including ERP, MIP-1, etc. PGE_2
Epithelial Cells	TGF-α, β Relaxin PGE IL-1
Endothelial Cells	PDGF Others?

Abbreviations used: PDGF, Platelet-Derived Growth Factor; EGF, Epidermal Growth Factor; TGF, Transforming Growth Factor; IL-1-6, Interleukin - 1-6; FGF, Fibroblast Growth Factor; TNF, Tumor Necrosis Factor; IGF, Insulin-like Growth Factor; IFN, Interferon; CSF, Colony Stimulating Factor; PGE_2, Prostaglandin E_2; ERP, Enzyme Releasing Protein; MIP-1, Macrophage Inflammatory Protein-1.

homeostatic conditions will be altered and the balance between the growth promoting and inhibiting activities of resident cytokines would no longer exist. Two possible mechanisms exist for disruption of homeostasis. One involves the epithelial cells, which after perturbation by an environmental agent may secrete chemotactic mediators resulting in an influx of various inflammatory cells into the lung from the bloodstream. An alternative mechanism involves perturbation of the concentration of substances that are already part of the homeostatic mechanism. An example of such a possibility is oxidative stress, which can result from direct exposure of lungs to

oxidants. Oxidative stress may also arise from the generation of oxidative agents by phagocytosis or other inflammatory cell processes that will directly alter the oxidative-reductive potential of the lung. For example, the glutathione content of lungs is decreased after treatment with several known toxicants. Such a decrease could result in activation of PDGF, which in turn serves as a growth promoting activity for fibroblasts and as a chemoattractant for various inflammatory cells. These inflammatory cells can, in turn, provide more PDGF and result in further stimulation of fibroblast cell proliferation, thereby amplifying an initial damaging event.

However, these changes can hypothetically be reversed after restoration of the normal homeostatic mechanism. The epithelial cell layers can be restored and the level of glutathione may return to the normal level. These activities, in turn, would inactivate PDGF activity and prevent further influx of inflammatory cells. However, depending on the specific types of inflammatory cells recruited to the lung, some of them will be able to directly injure new epithelial cells. Consequently, more chemoattractant may be secreted by injured epithelial cells, thereby further increasing the influx of inflammatory cells (Zigmond, 1989). Such a vicious cycle would create more damage in the lung and would increase the subsequent response of the lung, i.e. fibrosis. Thus, mechanisms of repair of this type of damage would depend upon the restoration of intact epithelium and reduced synthesis of chemoattractants, whereas progression of lung damage would be a consequence of failure to prevent further damage to the cells of the epithelial layer. This may be recognized as a restatement in contemporary terminology of a hypothesis first put forth by my colleague Dr. Hanspeter Witschi some years ago (Haschek and Witschi, 1979).

One of the consequences of repetitive injury would be to create the situation of uncontrolled proliferation of fibroblasts. It has been demonstrated that fibroblasts that normally do not produce PDGF become PDGF producers after repetitive stimulation. The nature of such a transformation is not completely understood. Such a transformation would result in uncontrolled growth of fibroblasts through a mechanism of paracrine/autocrine regulation. At this stage, the process of pulmonary fibrosis might well become irreversible.

These speculations suggest that cytokines, especially the competence factor PDGF, play an important role in the development of fibrogenesis. A similar mechanism would exist to explain the increased collagen content of the fibrotic lung. There is probably a correlation between fibroblast hyperplasia and the deposition of collagen in fibrotic lung. PDGF has as yet no known direct interaction with the collagen gene. However, there are a plethora of mediators in the lung that could participate in the regulation of collagen synthesis and secretion (Adams, 1989 . Ascorbate, estradiol, insulin, IGF-1, TGF-b, IL-1, and TNF-alpha have all been reported to increase the synthesis of type I collagen. On the other hand, glucocorticoids, vitamin D3, PTH, PGE2, alpha-IFN, certain tumor viruses and tumor promotors have all been reported to decrease collagen synthesis. Potential effects of cytokines on the post-translational metabolism of collagen are currently unknown. Clearly, any alteration of the proposed homeostasis in cytokine distribution would have indirect (at the level of regulation of fibroblast number and activity), and possibly direct, regional effects on the metabolism of collagen.

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Antioxidant Defense Mechanisms in Asbestos-Induced Lung Disease

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ABSTRACT

Several studies suggest that active oxygen species (AOS) are involved in the development of asbestos-induced lung diseases. Experiments in this laboratory have focused on antioxidant enzymes as preventive agents of asbestos-induced cell injury when added to cultures of alveolar macrophages (AMs), tracheobronchial epithelial cells, the progenitor cells of lung cancer (bronchogenic carcinoma), and lung fibroblasts, a cell type affected in asbestosis. Most recently, lung injury, inflammation, and pulmonary fibrosis have been ameliorated in an inhalation model of asbestosis using administration of antioxidant enzymes to rats during their exposure to asbestos. Current studies are focusing on the patterns and mechanisms of induction of antioxidant enzymes in the lung after inhalation of asbestos. These studies indicate that levels of antioxidant enzymes [total superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase] are increased in lungs within days after the initiation of exposure to high airborne concentrations (~ 7 mg/m^3 air) of crocidolite asbestos. Use of cDNA probes for CuZn and Mn-containing SODs indicates that steady-state mRNA levels of Mn-SOD are increased in the lungs of asbestos-exposed animals, while CuZn-SOD expression is unchanged. Results suggest that inhalation of crocidolite asbestos induces increased expression of an antioxidant enzyme in lung which is induced by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF) in a variety of cell types.

INTRODUCTION

Asbestos is a term for a group of hydrated silicate minerals that crystallize in a fibrous habit. The mechanisms of asbestos-induced occupational diseases are poorly understood; however, several studies suggest that AOS are elaborated in fiber-induced cell injury and lung disease (reviewed in Mossman and Marsh, 1989; Mossman et al., 1990a). These AOS may be released from AMs or polymorphonuclear leukocytes (PMNs) which accumulate in the airspaces or interstitium after inhalation of dusts. In addition, iron-containing fibers such as crocidolite asbestos generate AOS by redox reactions occurring on the fiber surface.

Key Words: Asbestos, active oxygen species, antioxidant enzymes, pulmonary fibrosis, superoxide dismutase

The lung is equipped with an elaborate defense system consisting of antioxidant enzymes and other naturally occurring scavengers of AOS such as ceruloplasmin and vitamin E (Heffner and Repine, 1989). Since the concentrations of asbestos fibers (~ 10^5 total fibers/gm wet lung) are considerable in the lungs of the general population (Churg, 1982), we have focused on the induction of antioxidant enzymes in lung as a possible mechanism of lung defense. We hypothesize that asbestos-induced cell injury and consequent disease occur at high airborne concentrations of fibers when the balance between oxidant generation and antioxidants in the lungs is altered, i.e., when the antioxidant defense system is overwhelmed. Specifically, we have focused on the expression and activity of antioxidant enzymes in an inhalation model of asbestos-induced lung injury and pulmonary fibrosis (Mossman et al., 1990b).

METHODS

Inhalation Protocols: Male Fischer 344 rats, weighing approximately 200 to 250 grams, were exposed to NIEHS crocidolite asbestos (7-10 mg/m^3 air) in a regimen resembling past occupational exposure to asbestos in humans. In brief, asbestos fibers were generated using a modified Timbrell dust generator. The fiber size distributions of aerosolized asbestos were determined on 200 fibers (Figure 1). Airborne concentrations of dusts were monitored on a daily basis. Asbestos-exposed animals were exposed for 6 hours per day, 5 days per week for 10 days. Sham control animals were placed in dust-free chambers and handled identically. At 1, 3, 6, and 9 days of exposure, and at 14 days after cessation of exposure, sham and asbestos-exposed rats [N=4 per group/experiment in triplicate experiments] were removed from the inhalation chambers. After intraperitoneal injection of pentobarbital, the chest cavity was opened, and the lungs perfused with heparinized calcium and magnesium-free, phosphate buffered saline via cardiac puncture. The left lung was removed, placed in liquid nitrogen, pulvarized in a mortar and pestle, and stored at -70°C for assays to determine antioxidant enzymes. The right lung lobes then were lavaged (Mossman et al., 1990b) before isolation of RNA.

Assays for Antioxidant Enzymes: Catalase was determined as described by Beers and Sizer (1952) measuring decomposition of H_2O_2 at 240 nm. Selenium-dependent glutathione peroxidase (GPX) was determined by the method of Paglia and Valentine (1967) measuring oxidation of NADPH at 340 nm and using H_2O_2 as substrate. Total SOD, which includes both CuZn-SOD and Mn-SOD forms was measured at pH 10.0 using SOD to inhibit cytochrome c reduction generated by a stable xanthine-xanthine oxidase reaction (Crapo et al., 1978). The protein content of lung tissue was assessed according to Bradford (1976). All enzyme activities were expressed per mg protein of lung. Results were evaluated using the "SPSX-program."

Northern Blot Analyses: Total RNA was extracted from perfused, lavaged lungs using the method of Chomczynski and Sacchi (1987). RNA then was denatured and fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel. To ensure that an equal amount of RNA was present in each lane of the gel, RNA was first spectrophotometrically quantified, reprecipitated, washed, and quantitated a second time before loading each lane with RNA (15 ug). RNA then was transferred to nitrocellulose and baked and hybridized with cDNA probes for Mn-SOD or CuZn-SOD (Y.-S. Ho, Raleigh, NC). A probe for 28s ribosomal RNA (pI19), obtained from Dr. K. Cutroneo, UVM Department of Biochemistry, was used as a control probe. Blots were washed and visualized by exposure to Kodak X-Omat AR film at -70°C using intensifying screens. Various species of mRNA were quantified using a Betascope blot analyzer (Betagen Corp., Waltham, MA).

RESULTS

Activity of Antioxidant Enzymes in Lungs of Asbestos-exposed and Sham Animals: Table 1 shows levels of antioxidant enzymes in the lung after short-term inhalation of crocidolite and at 14 days after cessation of exposure. Inhalation

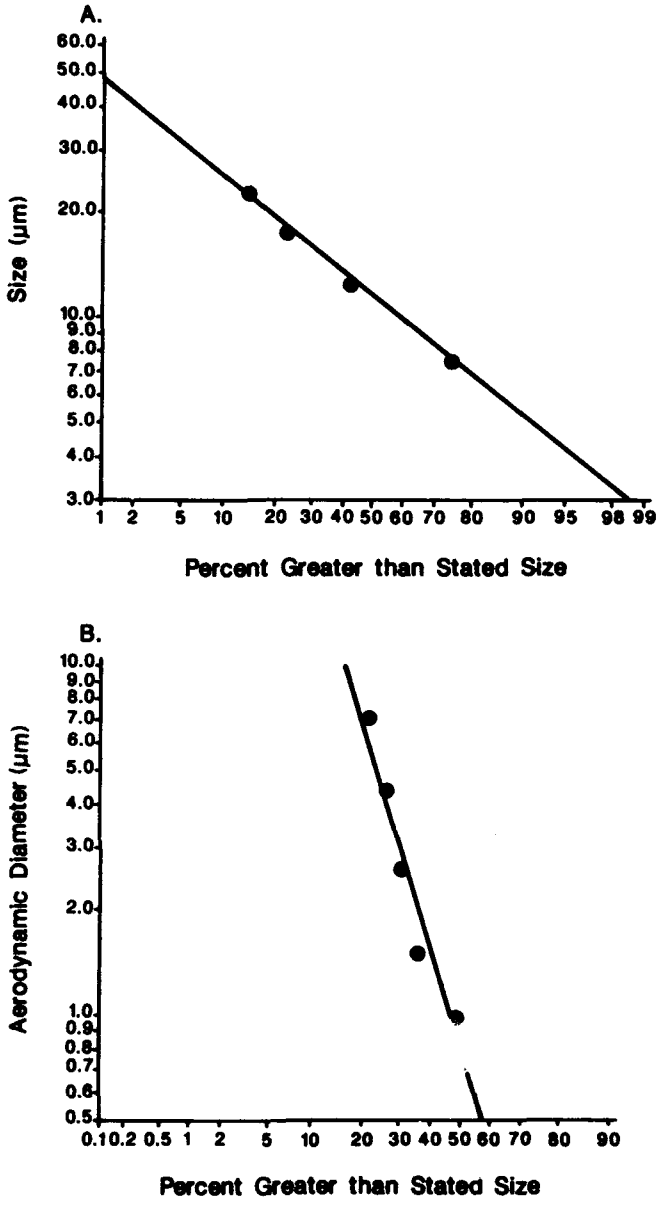

Figure 1. Size distributions of N.I.E.H.S. crocidolite asbestos after generation in an inhalation chamber. The lengths of 200 fibers were measured by scanning electron microscopy (A). Aerodynamic particle diameter was determined by cascade impaction (B).

of asbestos resulted in increases in all of the lung antioxidant enzymes examined, an observation supporting the results of our prior experiments showing an elevation of total SOD activity in tracheal epithelial cells exposed to either chrysotile or crocidolite asbestos in vitro (Mossman et al., 1986). The increase in total SOD activity in lung was most striking at 2 weeks after cessation of exposure. Activity of catalase was increased significantly after 6 and 9 days of exposure to asbestos, as well as 14 days after cessation of exposure. Similar trends were observed in the induction of GPX activity.

TABLE 1

Antioxidant Enzyme Activities in Sham and Crocidolite Asbestos-Exposed Rat Lungs at Various Time Periods During and Following (14 Days in Clean Air) a 10 Day Period of Inhalation

Enzyme activities are expressed as the Mean ± S.E.M. of triplicate experiments (N=11-12/group/time period). *Significantly increased ($p < .05$) in comparison to sham values.

	Total SOD (U/mg protein)		GPX (mU/mg protein)	
Days	Sham	Asbestos	Sham	Asbestos
1	86.0 ± 12.9	118.8 ± 27.6	650.4 ± 83.3	662.0 ± 142.2
3	116.9 ± 16.3	318.2 ± 115.8	620.8 ± 61.1	619.2 ± 85.1
6	112.8 ± 13	171.1 ± 27.6	639.8 ± 98.9	761.2 ± 91.5
9	113.1 ± 12.1	231.9 ± 54.7	576.6 ± 103.7	1108.2 ± 221.9*
10+(14)	208.4 ± 69.0	389.1 ± 73.5*	643.4 ± 131.4	1336.6 ± 290.6*

	Catalase (U/mg protein)	
Days	Sham	Asbestos
1	117.0 ± 16.9	148.2 ± 24.2
3	127.5 ± 15.4	182.9 ± 33.7
6	117.4 ± 11.2	184.5 ± 22.1*
9	141.2 ± 25.6	220.9 ± 32.4*
10+(14)	197.7 ± 25.9	266.9 ± 38.9*

Northern Blot Analyses: Using cDNA probes for Mn- and CuZn-containing SODs, we next examined steady-state mRNA levels in the lungs of sham rats and those exposed to crocidolite to determine if asbestos caused increased gene expression of certain isoforms of SOD in the lung. The Northern blot in Figure 2 shows mRNA species of Mn-SOD and CuZn-SOD in the lungs of sham and asbestos-exposed rats at 6 days after the initiation of exposure. Five species of mRNA for Mn-SOD occurred in the rat lung as has been reported recently for human lung (Wispe et al., 1989). Increases in Mn-SOD mRNA were observed in asbestos-exposed rats in comparison to controls while expression of CuZn-SOD mRNA was similar in both groups. This pattern has been observed at other time periods examined. Northern blots presently are being quantified using a Betascope blot analyzer (Betagen Corp.) to allow determination of statistical differences between groups (Janssen et al., in preparation).

DISCUSSION

Occupational exposure to asbestos is associated with the development of malignant (mesothelioma, lung cancer) and nonmalignant (asbestosis) diseases of the lung (Mossman and Gee, 1989). In an inhalation model of disease, a marked inflammatory response precedes pulmonary fibrosis and coincides with markers of lung injury in bronchoalveolar lavage (BAL), such as increased amounts of lactate dehydrogenase (LDH) and protein (Mossman et al., 1990b). Under these circumstances, enhanced lipid peroxidation also occurs in BAL cells and fluid (Petruska et al., 1989). Because exogenous administration of polyethylene glycol (PEG)-conjugated catalase to asbestos-exposed rats inhibits inflammation, lung injury and asbestosis, we have concluded that AOS are causally related to the development of asbestos-related pulmonary fibrosis. Thus, antioxidant defense enzymes in the lung may be overwhelmed at high airborne concentrations of fibers such as those used in experimental animal models of asbestosis. In support of this hypothesis, inhalation of crocidolite asbestos at high airborne concentrations (7-10 mg/m^3 air) causes an increase in enzyme activities of GPX, catalase, and total SOD (including both Mn- and CuZn-containing forms) in the unlavaged lung. In studies here, we examined lavaged lung tissues to determine

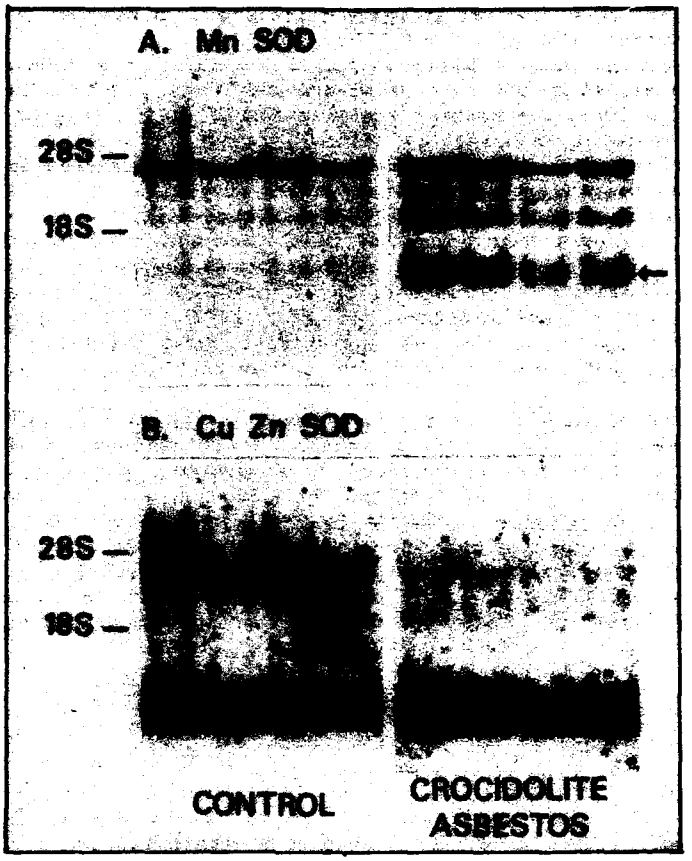

Figure 2. Northern blot analysis of Mn-SOD (A) and CuZn-SOD (B) in rat lung (N=4/group). Total RNA was isolated by procedures described in the text and 15 ug of RNA fractionated on an agarose-formaldehyde gel, blotted onto nitrocellulose and hybridized to ^{32}P-labeled cDNA probes. The 1 Kb species of Mn-SOD mRNA is indicated by arrow.

if increased enzyme activity of SOD in the asbestos-exposed lung reflected an increase in gene expression of either Mn-SOD or CuZn-SOD. Although both of these isoforms are encoded in the nucleus, Mn-SOD is found in the mitochondria and CuZn-SOD in the cytoplasm. Both enzymes scavenge superoxide (O_2), a radical initiating a cascade of active oxygen species. In the asbestos-exposed lung, increased enzyme activity of total SOD was accompanied by increased steady-state mRNA levels of Mn-SOD. In contrast, gene expression of CuZn-SOD was unaffected. Thus, increased total SOD activity in the lungs of asbestos-exposed rats appears to reflect an increased ratio of Mn-SOD to CuZn-SOD mRNAs after oxidant injury by asbestos. It should be emphasized that enzyme assays were performed on unlavaged lungs, thus the contribution of the inflammatory cells of the airspaces to the activity of antioxidant enzymes in asbestos-exposed lungs is unclear. Current experiments on enzyme determinations in lavaged lung tissues and immunocytochemical localization of Mn-SOD and CuZn-SOD in BAL cells and lung are designed to address this question.

Our results indicate that Mn-SOD and CuZn-SOD genes are not coordinately regulated in the lung in response to asbestos, an observation consistent with studies showing that inducibility of these enzymes differs in cells exposed to cytokines or lipopolysaccharide (LPS). For example, IL-1 and TNF cause increased expression of Mn-SOD in a number of cell types whereas expression of CuZn-SOD and other antioxidant enzymes are unchanged (Wong et al., 1989; Wong and Goeddel, 1988). A dramatic induction of Mn-SOD occurs in pulmonary epithelial cells in

response to TNF, IL-1, and LPS in vitro while steady-state mRNA levels of CuZn-SOD remain constant (Visner et al., 1990).

One important issue is whether cytokines modulate the increases in steady-state mRNA levels of Mn-SOD in mineral-exposed lungs. TNF is increased in the lungs of mice after intratracheal instillation of silica (Piquet et al., 1990) and in AMs exposed to asbestos or silica in vivo (Driscoll et al., 1990a,b). Whether cytokines are required for asbestos-induced gene expression of Mn-SOD in lung fibroblasts in vitro is currently under investigation in our laboratory. Another area of exploration is whether the increased expression and/or activity of antioxidant enzymes occurs in other inhalation models of pulmonary fibrosis. For example, levels of antioxidant enzymes are unchanged or decreased in rat lungs after inhalation of cristobalite, a nonfibrous silica causing massive inflammation and silicosis (Absher et al., 1989). These preliminary data indicate different mechanisms of antioxidant gene regulation and/or post-transcriptional modification of these antioxidant genes in asbestosis and silicosis.

Our results suggest that the lung mounts a response to airborne asbestos fibers via induction of antioxidant enzymes. At concentrations of asbestos used here, the lung is clearly overwhelmed by an overload phenomenon and fibrosis ensues. However, at low concentrations of asbestos, such as those encountered in the workplace today (i.e., $\leq$ .2 fibers/cc air, the current O.S.H.A. standard) asbestosis does not appear to develop in workers (Gaensler, et al. 1988). We are currently measuring oxidant and antioxidant enzyme activities in lung at lower airborne concentrations of crocidolite in an effort to determine whether antioxidant enzymes are induced at concentrations of asbestos where disease does not occur.

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Overload of Lung Clearance Is Associated with Activation of Alveolar Macrophage Tumor Necrosis Factor and Fibronectin Release

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ABSTRACT

This report summarizes recent findings on the relationships among overloaded lung clearance, activation of alveolar macrophages (AM) release of inflammatory mediators and the development of fibrosis using TiO2 as a model nuisance type dust. Briefly rats were intratracheally instilled with 2-100 mg TiO2/kg body weight and AM tumor necrosis factor or fibronectin release determined ex vivo 1, 7, 14 and 28 days after exposure. Lung dust burdens were determined 1 and 28 days after exposure. Histopathology was assessed 28 and 90 days after exposure. Intratracheal instillation of ≥50 mg/kg TiO2 resulted in overloaded lung clearance. TiO2 doses ≥50 mg/kg stimulated transient increases in AM TNF release and a persistent increase in AM fibronectin secretion. Histopathology demonstrated dose-related interstitial inflammation with fibrosis developing only after treatment with ≥50 mg/kg TiO2. Results from these studies suggest activation of AM secretory activity may play a key role in adverse pulmonary responses to high dust burdens of relatively innocuous materials. Studies investigating in vitro responses of AM to dust indicated that direct TiO2:AM interaction does not stimulate release of TNF or fibronectin, however, pre-exposure to γ-interferon can render AM responsive to TiO2 with respect to increased TNF release.

INTRODUCTION

The secretory activity of alveolar macrophages (AM), while important to lung defense, is also thought to play a key role in the pathogenesis of interstitial lung disease. A variety of studies have demonstrated that pneumotoxic dusts such as crystalline silica and asbestos can activate AM to release factors which recruit and activate inflammatory cells, as well as, stimulate fibroblast proliferation and collagen synthesis (Davis, 1986). Two factors whose release is increased after SiO2 or asbestos exposure are tumor necrosis factor (TNF) and fibronectin (Rom et al., 1986; Dubois et al., 1989; Driscoll et.al., 1990a). TNF is a 17 kd peptide which possesses a number of pro-inflammatory activities. Fibronectin, a 440 kd glycoprotein, is a chemotactic and growth factor for mesenchymal cells. Thus, both these macrophage-derived mediators have the potential to play an important role in pulmonary inflammation and tissue repair processes.

Key Words: alveolar macrophage, tumor necrosis factor, fibronectin, fibrosis, titanium dioxide, clearance overload

In contrast to SiO2 and asbestos, nuisance-type dusts, under most circumstances, are not associated with adverse pulmonary responses. However, exposure to relatively innocuous dusts can result in chronic inflammation and fibrosis when pulmonary dust burdens are reached which overload normal particle clearance mechanisms (Morrow, 1988; Oberdorster, 1988). Chronic inflammation and fibrosis have been observed at excessive lung burdens of titanium dioxide (TiO2), diesel exhaust and test toner; materials thought to possess a low degree of toxicity (Lee et al, 1986; Wolff et al., 1987; Muhle et al., 1988). At present, there is limited information on similarities and/or differences in mechanisms underlying the pulmonary responses to highly toxic dusts versus relatively innocuous dusts under conditions of clearance overload.

This manuscript summarizes some of our recent findings on activation of alveolar macrophage secretory activity after exposure to excessive lung burdens of TiO2, a material generally regarded as a nuisance-type dust. Results presented demonstrate that at TiO2 lung burdens which overload clearance, AM become activated to release TNF and increased levels of fibronectin, responses associated with an infiltration of inflammatory cells and development of fibrosis, respectively. Additionally, results from in vitro exposure of AM to TiO2 or SiO2 indicate the lung environment plays a key role in AM activation under conditions of overloaded clearance and suggest γ-interferon may represent one factor which is important in rendering AM susceptible to activation by TiO2.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Fischer 344 rats weighing 180-200g (Charles River Breeding Laboratories, Kingston, NY) were housed in an air conditioned room (25°C, 50% RH) on a 12 hour light/dark cycle. Animals were provided Purina Rodent Chow (5001) and tap water ad libitum throughout the study.

Dust Exposures for Bronchoalveolar Lavage Studies

The general experimental design and intratracheal instillation procedure have been described previously (Driscoll et al., 1990a). Briefly, animals were intratracheally instilled with sterile saline (control group) or saline suspensions of TiO2 (Anatase; Fisher Scientific, Fair Lawn, NJ). TiO2 was heated to 200°C for two hours for sterilization prior to use. The particle size (Feret's diameters) determined microscopically after 15 minutes of sonication was 2.1 ± 1.5 μm and surface area determined by nitrogen adsorption was 8.8 m^2/g. Instillations were performed at a dosing volume of 1.0 ml/kg body weight with TiO2 doses of 5, 10, 50, and 100 mg/kg body weight. Intratracheal instillations were performed on lightly anesthetized animals (Na pentabarbital, intraperitoneal injection; 3 mg/kg) using a pediatric laryngoscope and a #20 ball tipped dosing needle attached to a 1 ml syringe placed 1 cm into the trachea.

Bronchoalveolar Lavage and Cell Culture

At 1, 7, 14, and 28 days post-instillation 5 or 6 animals/treatment group were sacrificed by intraperitoneal injection of Na pentobarbital (50 mg/kg) and exsanguination via the abdominal aorta. Bronchoalveolar lavage (BAL) and AM culture were performed as described previously (Driscoll et al., 1990a). Briefly, the trachea was cannulated and the lung infused 6 times with Ca/Mg-free phosphate buffered saline solution (pH=7.2; PBS) at a volume of 8 ml/wash. The bronchoalveolar lavage fluid (BALF) was centrifuged at 300 x g for 10 minutes, and the cell pellet resuspended in RPMI 1640 (Gibco, Grand Island, NY) containing 25 mM HEPES buffer. BALF cell number and viability were determined by hemocytometer counting and trypan blue exclusion, respectively, and cell differentials were performed on cytocentrifuge preparations, fixed in methanol and stained with Diff Quik™ (American Scientific). The BALF cell suspensions

were adjusted to a concentration of 1 X 10^6 viable AM/ml and one ml seeded into 35mm plastic tissue culture dishes. AM were allowed to adhere for 60 minutes in a humidified incubator (37°C and 5 % CO_2) after which adherent monolayers were rinsed vigorously 3 times with RPMI 1640 media. The rinsed monolayers were incubated (37°C and 5% CO_2) for 24 hr in 1 ml RPMI 1640 containing 2 mg/ml fraction V bovine serum albumin (BSA; Sigma). The AM conditioned media was filtered (0.45 µm pore size) to remove nonadherent cells and frozen at -70°C until analysed for the presence of TNF or fibronectin. TNF bioactivity was determined using an L929 cell lysis assay and immunoreactive fibronectin was determined by ELISA as described previously (Driscoll et al., 1990a, b).

Histopathology

On days 28 and 90 (at 100 mg/kg, day 60) after instillation, five animals/treatment group (at 100 mg/kg, 3 animals/treatment) were sacrificed by ether inhalation and exsanguination via the abdominal aorta. The lungs were infused to 25cm H_2O with 10% buffered neutral formalin. Paraffin embedded sections from both lungs were stained with hematoxylin and eosin (H&E) or Masson's trichrome for light microscopic examinations.

In Vitro Exposure of AM

Methods used for in vitro exposure of F344 rats alveolar macrophages are described in detail elsewhere (Driscoll et al., 1990b and c). Briefly, AM were obtained by bronchoalveolar lavage of untreated F344 rats, suspended in RPMI 1640 media and 5 x 10^5 cells/well seeded for experiments examining TNF release and 2 x 10^6 AM/well seeded for experiments examining fibronectin release. Heat sterilized (200°C x 2 hr) SiO2 or TiO2 was suspended at concentrations of 10, 30, 100, or 300 µg/ml in RPMI 1640 containing 2 mg/ml BSA and 1 ml of the dust suspension added to cultures of adherent AM for 48 hr. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2. AM conditioned media was analysed for several consituents including LDH, TNF and fibronectin. The experiment was repeated 3 times, using cells pooled from at least 5 animals. Additional experiments were conducted to examine the effects of 24 hr pretreatment with 500 units/ml rat γ-interferon on TNF release upon a subsequent 24 hr exposure to 100 µg/ml SiO2 or, 100 and 1000 µg/ml TiO2.

Pulmonary Dust Retention

Groups of 15 rats were intratracheally instilled as described above with TiO2 at 2, 5, 10, 50, and 100 mg/kg body weight. Five animals/group were sacrificed at 1, 7, and 28 days after treatment, the lungs removed enbloc, trimmed, weighed and lyophylized. Titanium levels in lung tissues were determined as described previously (Driscoll et al., 1990b).

Statistical Analysis

All data are expressed as actual values except dust retention data which are presented as a percentage of the day 1 group mean lung burdens. Differences between treatments were evaluated using a one-way ANOVA, followed by pairwise comparisons using the Newman-Keuls test (Zar, 1984). Statistical significance was considered at $p<0.05$.

RESULTS

The lung burdens determined 24 hr after instillation of 2, 5, 10, 50 or 100 mg/kg TiO2 were 0.4 ± 0.1, 1.1 ± 0.2, 2.0 ± 0.3, 8.8 ± 0.5, 15.2 ± 0.5 mg TiO2/g lung weight, respectively. Dust retention 28 days after instillation as a

percentage of the day 1 lung burdens are shown in Figure 1. Instillation of 50 and 100 mg/kg TiO2 resulted in increased dust retention relative to that observed for instilled doses of 2, 5, or 10 mg/kg.

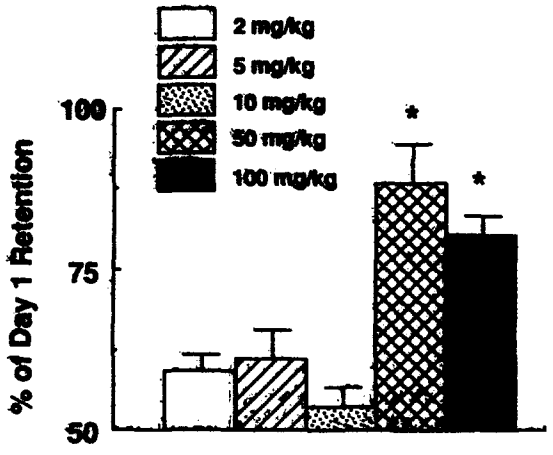

Figure 1. Retention of TiO2 28 days after instillation. Results are presented as a percentage of lung burdens determined 24 hr post exposure, X±SE; N=5. (*) denotes a significant difference from 2, 5, and 10 mg/kg dose groups; $p<0.05$.

Principal histological changes 28 days after TiO2 treatment consisted primarily of particle laden macrophages and interstitial inflammation with responses 90 days after exposure being of similar or decreased severity relative to those at day 28. Masson's trichrome stained sections were evaluated for increased prominence of collagen, which was interpreted to reflect fibrosis. At 28 days no fibrosis was observed for TiO2 exposed rats, however, at the later times fibrosis was present at TiO2 doses ≥50 mg/kg.

The effects of dust exposure on AM release of TNF or fibronectin are described in detail elsewhere (Driscoll et al., 1990a and b) and summarized with histopathology (collagen staining) and lung burden data in Table 1. TiO2 at doses of 50 or 100 mg/kg stimulated transient increases in AM TNF release, with lower dose levels eliciting no significant TNF response. Instillation of 50 or 100 mg/kg TiO2 stimulated AM to release increased levels of fibronectin, in contrast, exposure to 10 mg/kg elicited only a transient (day 7) increase in fibronectin release and treatment with 5 mg/kg did not stimulate a fibronectin response.

The effects of in vitro SiO2 or TiO2 exposure on release of LDH, TNF and fibronectin are summarized for doses of 30 and 300 µg/ml in Table 2. SiO2, but not TiO2, resulted in increased LDH as well as TNF release. In contrast neither dust had a significant effect on fibronectin secretion.

The effects of γ-interferon pretreatment on dust-induced AM TNF release are shown in Figure 2. Treatment with γ-interferon alone elicited small but significant increases in AM TNF release. Pretreatment with γ-interferon followed by SiO2 resulted in an increase in AM TNF release which was additive (equal to SiO2 alone plus γ-interferon alone). In contrast, combined treatment with 1000 µg/ml TiO2 and γ-interferon resulted in a stimulation of TNF release which was synergistic. Treatment of γ-interferon primed cells with 100 µg/ml TiO2 did not stimulate TNF release over that elicited by γ-interferon alone.

DISCUSSION

A number of studies have demonstrated that lung burdens of low solubility dusts which overload normal lung clearance mechanisms are associated with development of chronic pulmonary inflammation, fibrosis and in some instances lung tumors (Morrow, 1988; Oberdorster, 1988). Our laboratory has been investigating mechanisms underlying the pathogenesis of dust-induced interstitial lung disease. In the present report we summarize our recent observations on activation of AM secretory activity after exposure of rats to high and low lung burdens of TiO2, a material generally considered to be a nuisance-type dust. The results discussed demonstrate an association between overload of lung clearance, stimulation of AM

TABLE 1.

Pulmonary Responses to Intratracheally Instilled TiO_2 Relative to a Saline Instilled Control Group: Macrophage TNF and Fibronectin Release, Dust Retention, and Development of Pulmonary Fibrosis[a].

TiO2 Dose	AM TNF Release[b]				AM Fibronectin Release[b]			Dust Retention	Fibrosis	
	day 1	day 7	day 14	day 28	day 7	day 14	day 28	day 28	day 28	day 90[c]
100 mg/kg	+	-	+	-	+	+	+	increased	-	+
50 mg/kg	-	+	+	-	+	+	+	increased	-	+
10 mg/kg	-	-	-	-	+	-	-	normal	-	-
5 mg/kg	-	-	-	-	-	-	-	normal	-	-

[a] changes in dust retention are relative to TiO2 retentions observed for dose groups ≤10 mg/kg

[b] (+) = statistically significant increase above control; (-) not statistically different from control

[c] day 60 for 100 mg/kg groups

TABLE 2.

Response of Rat Alveolar Macrophages to *In Vitro* Treatment with SiO2 and TiO2 (N=3; X±SE)

Treatment	Lactate Dehydrogenase (units/10^6cells/24 hr)	Tumor Necrosis Factor (units/10^6cells/24 hr)	Fibronectin (ng/10^6cells/24 hr)
Control	1.9 ± 1.4	<2	67.1 ± 7.7
SiO2:			
30 µg/ml	7.3 ± 0.3 *	12.2 ± 2.2 *	68.8 ± 3.9
300 µg/ml	26.0 ± 0.3 *	52.2 ± 5.3 *	68.2 ± 4.6
TiO2:			
30 µg/ml	0.8 ± 0.2	<2	75.6 ± 13.1
300 µg/ml	1.3 ± 0.2	<2	56.9 ± 9.6

* significantly different from control; $p<0.05$

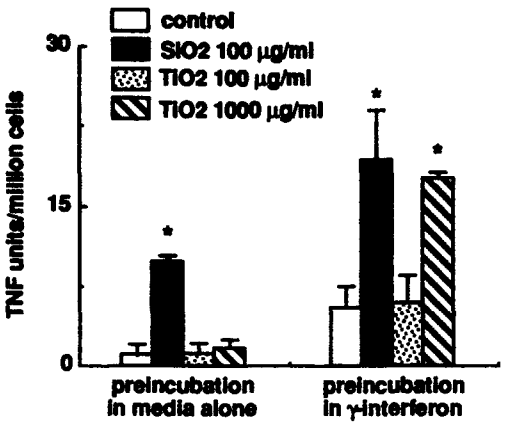

Figure 2. Effect of a 24 hr pretreatment with 500 u/ml rat γ-interferon on TiO2 and SiO2-induced AM TNF release. Data are presented as the X±SD, N=3. (*) denotes statistically significant difference from appropriate non-dust exposed control; $p<0.05$.

to release TNF and fibronectin, and the eventual development of fibrosis. The in vitro data presented indicate that the pulmonary environment plays a key role in activation of AM after dust exposure and suggests γ-interferon represents one factor with the potential to render AM susceptible to activation by TiO2.

To further elucidate the mechanisms underlying the activation of inflammatory processes in the lung after mineral dust exposure, we have focused our attention on the AM. This cell, because of its ability to release mediators which can modulate the activities of inflammatory and immunocompetent cells, has the potential to play a key role in the initiation and perpetuation of inflammatory responses within the lung. TNF represents an AM-derived cytokine with pro-inflammatory activities. This cytokine can stimulate expression of adhesion molecules for inflammatory cells on capillary endothelium (Bevilacqua et al., 1989); activate release of neutrophil and monocyte chemotactic peptides by a variety of cells including macrophages, fibroblasts and endothelial cells (Larsen et al., 1989; Strieter et al., 1989); and stimulate phagocytic cells to release reactive oxygen species and lysosomal enzymes (Klebanoff et al., 1986; Tsujimoto et al., 1986). Thus, this cytokine has the potential to play a key role in the recruitment and activation of inflammatory cells to the lung after inhalation of noxious materials. More recently, the key role of TNF in lung fibrosis was demonstrated by Piguet et al. (1989, 1990) who reported that treatment of SiO2 or bleomycin exposed mice with anti-TNF antibodies markedly attenuated collagen deposition. In the present report we summarize results suggesting a positive association between intratracheally instilled doses of TiO2 which overload lung clearance and activation of AM TNF release. Interestingly, statistical evaluation on an individual animal basis of the relationship between dust-induced increases in ex vivo AM TNF release and numbers of neutrophils in bronchoalveolar lavage fluid reveal a significant positive correlation (Driscoll et al, 1990a). This association strongly suggests a role for TNF in the recruitment of inflammatory cells under conditions of excessive dust exposure. Given the known pro-inflammatory activities associated with this cytokine it is likely that TNF, at least in part, plays a role in the inflammation and fibrosis we observed at excessive lung burdens of TiO2.

In addition to TNF, AM can release fibronectin, a mediator with the potential to influence development of pulmonary fibrosis. Fibronectin is chemotactic for fibroblasts (Postlethwaite et al., 1981); can mediate interactions between a variety of cell types and the extracellular matrix (Mosher, 1984; Macarak and Howard, 1983); and, acting in concert with other growth factors can stimulate fibroblasts to proliferate (Bitterman et al., 1983; Bitterman et al., 1986). Thus, fibronectin is thought to play a critical role in tissue repair; functioning to recruit cells to sites of tissue injury and inflammation, facilitating cell attachment to extracellular matrix proteins and promoting local cell proliferation. Excessive TiO2 lung burdens stimulated release of fibronectin, a response which persisted through the 28-day post-instillation period. In contrast, dust burdens which did not overload lung clearance elicited only transient or no increase in fibronectin release.

Importantly, persistent increases in fibronectin release were associated with eventual increases in collagen deposition (i.e., fibrosis). In this respect, previous studies have demonstrated that individuals with silicosis, asbestosis or coal worker pneumoconiosis have AM populations releasing increased levels of fibronectin (Rom et al., 1986) as well as increased fibronectin deposition at sites of pneumoconiotic lesions (Wagner et al., 1982). The prospective nature of our observations on increased fibronectin release and collagen deposition further support an important and early role for AM-derive fibronectin in the development of pulmonary fibrosis and suggest similarities in mechanisms of pulmonary fibrosis exist between highly toxic dusts and excessive lung burdens of relatively innocuous materials.

The mechanisms by which TiO2 stimulates AM to release TNF and fibronectin are unknown, however, results from our in vitro experiments indicate the pulmonary environment plays a key role. In this respect, the response seen after dust instillation could involve cells and or secondary mediators not present under in vitro exposure conditions. One cytokine, released by activated lymphocytes, which can "prime" macrophages to become more responsive to subsequent stimulation is γ-interferon (Adams and Hamilton, 1984). Since we previously observed that high doses of TiO2 result in persistent increases in BALF lymphocyte numbers (Driscoll et al., 1990a), and thus, potentially increased lymphocyte secretory products, we investigated the effects of in vitro γ-interferon pretreatment on responsiveness of AM to TiO2. These results indicated γ-interferon can prime AM to release TNF upon in vitro exposure to high doses (1000 μg/ml) of TiO2. It is noteworthy that the priming response was only observed for the high dose of TiO2, suggesting that this effect may be unique to AM which become engorged with particulate materials such as occurs in vivo under conditions of overloaded clearance. Interestingly, a similar response was not apparent for SiO2 exposure which suggests differences may exist in the mechanism of SiO2 and TiO2-induced TNF release. These findings demonstrate that secondary factors can increase responsiveness of AM to dust exposure and suggest γ-interferon has the potential, if released upon in vivo dust exposure, to influence AM responsiveness to TiO2.

In conclusion, the findings summarized in this report suggest that similar to highly toxic dusts (e.g., SiO2 and asbestos), the development of interstitial lung disease in animals exposed to excessive lung burdens of nuisance-type dusts may, at least in part, result from activation of AM to release pro-inflammatory factors. The correlation, observed between ex vivo AM TNF release after instillation of TiO2 and neutrophil numbers in BAL fluid clearly suggests a role for this cytokine in dust-induced recruitment and activation of inflammatory cells. Further, the association of persistent AM fibronectin release with the development of fibrosis provides additional support for this mediator in the pathogenesis of interstitial lung disease. Lastly, the in vitro studies indicate the pulmonary environment plays an important role in the activation of AM TNF and fibronectin release after exposure to excessive lung burdens of TiO2 and, suggests that factors such as γ-interferon could be important in modulating AM responsiveness to dusts in vivo.

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Interspecies Comparison of Lung Clearance of "Insoluble" Particles

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ABSTRACT

Lung clearance studies after the inhalation of monodisperse, radiolabelled test particles including lung retention measurements and excretion analysis allow for estimates of the kinetics of long-term particle transport out of the thorax into the gastro-intestinal tract. Data of several interspecies comparisons using either radiolabelled fused aluminosilicate particles or $^{57}Co_3O_4$ particles were reviewed and compared. Species included were: man, baboon, beagle dog, guinea pig, HMT rat, F-344 rat, Long-Evans rat, hamster, mouse.

Particle transport M(t) after the first days after inhalation is a slow clearance mechanism which is independent of the particle material and size used (0.5 - 4 µm geom. diameter). M(t) was reproducible in the experimental species studied. In man, baboon, and dog the initial daily fraction M_0 of the contemporary lung burden transported out of the thorax is 0.001 d^{-1} which is an order of magnitude less than the initial rates in rodents. Particle transport rate decreases rapidly from its initial value in all species studied. The decay of particle transport varies considerably between the species and strains. The half-life of the decreasing transport rate is slower in man, dog, F-344 rat, hamster and mouse (100 - 200 days) than in baboon, HMT rat and Long-Evans rat (< 50 days). From these studies estimates of lung retention during chronic aerosol exposure showed no equilibrium value indicating that long-term particle transport is not a sufficiently effective clearance mechanism to keep the lung burden from continuousy increasing during chronic exposure.

INTRODUCTION

Interspecies comparisons of lung clearance including human studies provide a potential tool to estimate the relevance of results obtained from experimental animal species with respect to their extrapolation to the human lung. In this paper data obtained from a few interspecies comparisons and studies on lung clearance of inhaled test aerosol par-

Key words: alveolar clearance, particle transport, interspecies comparison, monodisperse radiolabelled aerosol particles

ticles are reviewed with a special emphasis on the removal of intact particles from the lungs.

Aerosol particles deposited in the lungs are subject to two major clearance mechanisms out of the thorax depending on physical and chemical properties of the particles: mechanical transport M(t) of entire particles and/or translocation S(t) of dissolved material from the particle. Both clearance mechanisms are believed to be time dependent and competitive and independent (Cuddihy, 1984,1988). This differentiation of particle clearance from the lung parenchyma applies to man and a variety of experimental animal species (Bailey et al., 1989). The total rate of clearance $\lambda(t)$ is a fraction of the contemporary lung retention L(t):

$$\lambda(t) = - \frac{dL(t)/dt}{L(t)} \qquad \lambda(t) = M(t) + S(t) \qquad (1)$$

Soluble particles or soluble compounds of particles are mainly cleared by absorptive mechanisms consisting of transepithelial permeation and subsequent elimination via the blood. However, the dissociated material might bind to distinctive constituents of the various lung and cell fluids which will be translocated or retained in the lungs at time constants specific for these constituents. As a result, water soluble aerosol particles will disintegrate readily in the epithelial lining fluid but the material may or may not be cleared from the lungs.

It is generally recognized that particles which are not dissolved in the epithelial lining fluid, are phagocytized by alveolar macrophages within a few hours (Brain, 1985). Hence, long-term clearance from this region must involve these cells. Particles may be moved by macrophage mobility to the ciliated conducting airways to be cleared by mucociliary transport. Macrophage migration is effected by chemotactic and other biochemical factors, as was reviewed recently by Oberdörster (1988). Particles also will be transported across the epithelial barrier to be stored in interstitial tissue or to be carried on for storage in tracheobronchial and bifurcational lymph nodes (TBLN).

Moreover, particles which are negligibly soluble in water might be cleared from the lungs by translocation of dissolved particle material (Kreyling et al., 1986,1988, Bailey et al., 1989). Since the retained particles are incorporated in phagolysosomal vacuoles of alveolar macrophages on the epithelium almost all the time, the solvent is the aqueous vacuolar sol which also contains oxygen radical species, lysosomal, proteolytic and other enzymes, mediators, chelators and protons at a pH of about 5. Therefore, this solvent is different from extracellular lung fluids which eventually results in a more effective dissolution of various particle compounds in the lungs. Clearance of long-term retained particles out of the thorax by particle transport and translocation of dissolved particle material is shown schematically in Figure 1.

MECHANICAL PARTICLE TRANSPORT

The kinetics of mechanical particle transport from the alveolar region via the mucociliary escalator to the larynx cannot be determined directly. From lung retention measurements, total lung clearance is determined which is the sum of the clearance mechanisms from the alveolar region and from the tracheobronchial tree and the extrathoracic airways which might be superimposed even after extended times of retention (Gore et al., 1982; Stahlhofen et al. 1980). However, for this investigation data of fast clearance during the first few days after inhalation were excluded. Thereafter, it was assumed that

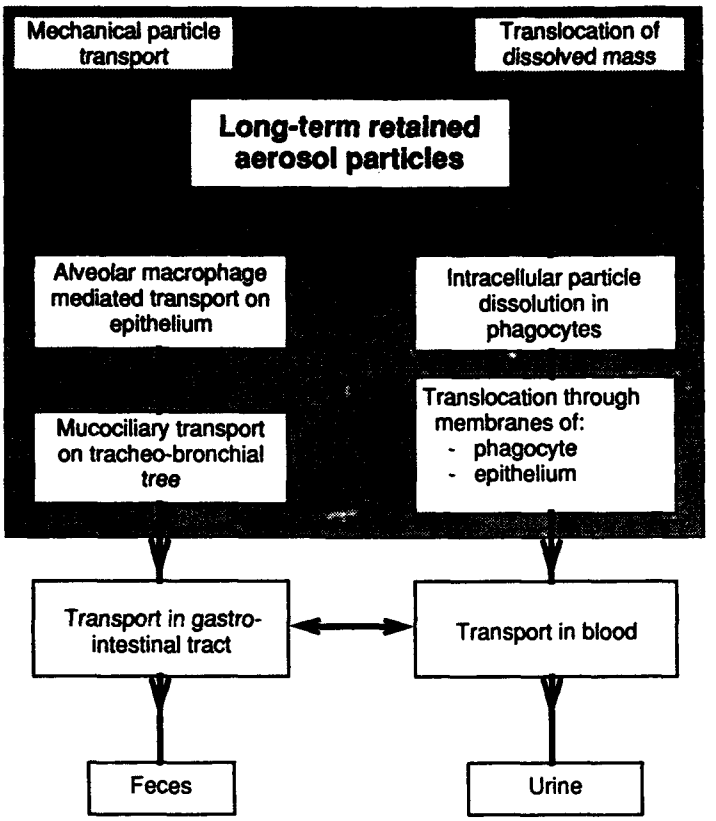

Figure 1. Lung clearance of long-term retained particles out of the thorax by particle transport and translocation of dissolved particle material.

long-term clearance from the tracheobronchial tree and the extrathoracic airways is a minor fraction of long-term clearance from the alveolar region.

According to equation 1, M(t) can be evaluated from lung retention measurements as long as S(t) is negligible, i.e. the test particles are negligibly soluble in the lungs over the entire time of observation. It is emphasized, however, that in vivo dissolution might be different from in vitro dissolution in simulant solvents (Lundborg et al., 1984; Kreyling et al., 1986,1988,1990b). Moreover, translocation from the lungs might vary between the species (Bailey et al., 1989). Although it is not necessarily required to study clearance of monodisperse test particles, their use is desirable to test the size dependence of particle transport. Additionally, particle dissolution and hence, translocation, becomes more complicated since particle dissolution extracellularly and intracellularly is particle size dependent (Mercer, 1967; Moss and Kanapilly, 1980; Kreyling et al., 1990a,1990b) resulting in erroneous estimates of particle transport. Therefore, this review is restricted to interspecies comparisons of lung clearance using monodisperse test particles.

In the past several investigators have used Fe_2O_3 test particles labelled with various radio-isotopes in human or experimental animal lung clearance studies (Albert et al., 1967, Morrow et al., 1967a,1967b; Bellmann et al., 1983,1986; Muhle et al., 1988). Translocation S(t) of dissociated Fe from these particles was generally difficult to determine due to the metabolism of Fe. However, long-term retention of dissociated Fe in the lungs and in other organs could not be ruled out. Hence, neither exclusive particle retention in the lungs nor estimates of S(t) were given excluding a proper estimation of M(t).

Using magnetic Fe_3O_4 particles, retention in the lungs was determined by magnetopneumography in man and experimental animals (Cohen et al., 1979; Oberdörster et al., 1984; Kalliomäki et al., 1985; Freedman et al., 1988). From these data total particle clearance was calculated but the evaluation of the clearance mechanism of particle transport M(t) would have required knowledge of S(t). Sensitivity of the magnetometer was another limitation of this approach, still requiring a rather high dose of retained magnetic particles. Since high lung burdens of test particles and/or apparently toxic particles might effect phagocytic and migratory functions of alveolar macrophages, particle transport might also be altered (McClellan et al., 1982,1986; Vostal et al., 1982). Therefore, the deposited dose of the test particles should be as low as possible and the material should not be specified to be cytotoxic.

A better means to estimate the kinetics of mechanical particle transport was the combination of lung retention measurements and excretion analysis after the inhalation of more or less insoluble, radiolabelled test particles (Snipes et al. 1983; Bailey et al. 1985a, 1985b, 1989, Kreyling et al., 1986+1988). This method makes use of the fact that particles which had been transported to the larynx are subsequently swallowed into the gastro-intestinal (GI) tract and are eventually excreted in feces. The amount of excreted particles in the feces and the amount of retained particles in the lungs were determined by radioactivity measurements from which the particle transport rate was determined. However, the analysis was complicated by the fact that a fraction of the particle material and/or the radiolabel might have been absorbed during passage through the GI-tract which might have been redistributed systemically and not excreted in feces. Another complication arose from the other clearance mechanism of translocation, i.e. a fraction of the particle material which was dissociated and translocated from the lungs to blood might have entered the GI-tract and was excreted in the feces.

Recently, the importance of translocation of dissolved particle material from the lungs to blood was clearly demonstrated for ^{57}Co-labelled fused aluminosilicate particles (^{57}Co-FAP) which were considered to be almost insoluble (Kreyling et al., 1988).
During a three year clearance study with beagle dogs not only lung retention measurements and excretion analysis was carried but also a chemical procedure was involved which separated ^{57}Co-FAP from non-particulate ^{57}Co in fecal samples. From these measurements and a supplementary study for the absorption of the radiolabel from FAP during passage of the GI-tract after gavage, particle transport M(t) was evaluated. The initial particle transport rate was 0.0006 d^{-1} and decreased monotonically with a rate of 0.004 d^{-1} (half-life 170 d). Since S(t) was 0.0005 d^{-1} and constant over the entire period it became the predominant clearance mechanism from the lungs after one year. The fraction of non-particulate ^{57}Co in fecal samples increased from an initially minor fraction to a similar amount as the ^{57}Co-FAP fraction, since 5% of the translocated non-particulate ^{57}Co circulating in blood was not excreted in urine but in feces.

Bailey et al. (1989) proposed a simple model (Figure 2) taking these metabolic effects into account from which particle transport was evaluated:

$$U(t) = b_u\, S(t) + g_u\, M(t) \qquad F(t) = g_f\, M(t) + b_f\, S(t) \qquad (2)$$

where U(t) and F(t) were the urinary and fecal excretion rates, i.e. the amounts of radioactivity excreted per day as fractions of the contemporary lung content L(t); b_u and b_f were the fractions of the radiolabel in urine and feces, respectively, following translocation of the radiolabel from the lungs to blood; and g_u and g_f were the fractions of the radiolabel excreted in urine and feces respectively, after particles had entered the GI tract. The transfer coefficients b_u, b_f, g_u, g_f were determined by supplementary excretion analyses after the radiolabel was injected intravenously or the test particles were ingested. Thus:

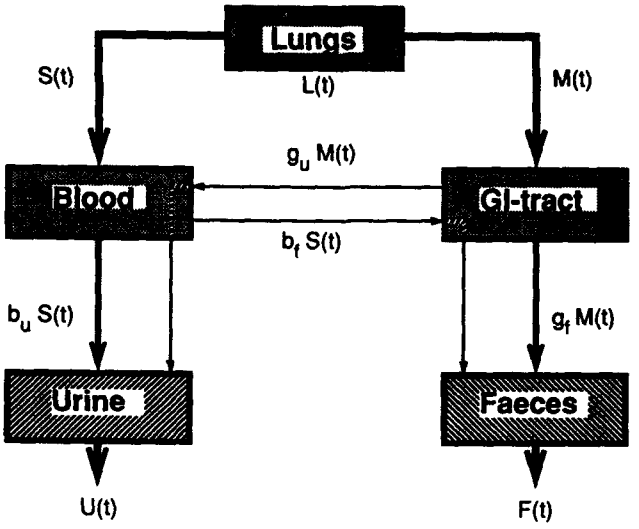

Figure 2. Transfer coefficients for the radiolabel following inhalation of test particles, showing the fractions of lung content L(t) cleared per day by mechanical particle transport M(t) and translocation of dissolved particle material S(t) at time t.

$$S(t) = \frac{g_f U(t) - g_u F(t)}{b_u g_f - b_f g_u} \qquad M(t) = \frac{b_u F(t) - b_f U(t)}{b_u g_f - b_f g_u} \qquad (3)$$

This model allowed systemic uptake of the radiolabel by other organs but it did not take into account clearance from these organs since it was assumed that rate constants for those organs would be negligibly small to effect the transfer coefficients b_u, b_f, g_u, g_f.

INTERSPECIES COMPARISON OF PARTICLE TRANSPORT

Data Base

There were a few interspecies comparisons of lung clearance which were based on this clearance model and the appropriate measurements. In these studies monodisperse test particles of two different materials were used: FAP labelled with various radiolabels and ^{57}Co labelled Co_3O_4 particles. Lung retention L(t) and both clearance mechanisms M(t) and S(t) of FAP were estimated in the following species:

man:	Bailey et al. 1985b
beagle dogs, Fischer-344 rats, CD-1 mice:	Snipes et al. (1983)
Hartley guinea pigs:	McClellan et al. (1984)
beagle dogs :	Kreyling et al., 1988
HMT rats, Syrian golden hamsters (DSN):	Bailey et al. 1985a

In a joint European attempt (Bailey et al., 1989, summary) organized by the European Late Effects Project Group (EULEP) of the Commission of the European Communities lung retention L(t) and both clearance mechanisms M(t) and S(t) of two different sizes of monodisperse, porous $^{57}Co_3O_4$ particles were evaluated and compared between:

man:	Foster et al., 1989, Pearman et al., 1990;
baboon:	André et al., 1989;
beagle dog:	Kreyling et al., 1989a;
Harwell guinea pig, HMT rat, Syrian golden hamster (DSN):	Collier et al., 1989;
Fischer-344 rat (SPF):	Patrick et al., 1989;
Sprague-Dawley rat:	Drosselmeyer et al., 1989;
mouse (CBA/H):	Talbot et al., 1989).

Particles from the same two batches were also used to determine clearance in Long-Evans rats. Additionally, lung clearance of monodisperse solid $^{57}Co_3O_4$ particles was investigated in baboons, beagle dogs and HMT rats (Kreyling et al.,1988,1989b; Collier et al., submitted). The solid $^{57}Co_3O_4$ particles were chosen to confirm that translocation S(t) in each species was proportional to the specific surface area of the particles. It also was chosen to prove reproducibility of particle transport M(t). Particle parameters are summarized in Table 1. Also incorporated in this interspecies comparison is another hu-

Table 1.

Parameters of monodisperse test particles and number N of subjects (animals) of each species for the interspecies comparisons of lung clearance.

Species / strains	fused aluminosilicate particles N	radiolabel	d_{geom} (µm)	$^{57}Co_3O_4$ particles N	density	d_{geom} (µm)
Man	13	^{88}Yt	4.0	4	porous	1.7, 0.8
	13	^{85}Sr	1.0			
Baboon				4	porous	1.7, 0.8
				2	solid	0.9
Beagle dog	2	^{57}Co	1.5	4,(8)*	porous	1.7, 0.8
	120	^{134}Sr	0.5, 1.0, 1.9	4	solid	1.6, 0.9
Guinea pig	49	^{134}Sr	1.3	24	porous	1.7, 0.8
HMT rat	30	^{85}Sr	1.2	60,(24)*	porous	1.7, 0.8
				20	solid	0.9
F-344 rat	320	^{134}Sr	0.5, 1.0, 1.9	44	porous	1.7, 0.8
Long-Evans rat				8	porous	1.7, 0.8
Syrian hamster	30	^{85}Sr	1.2	60	porous	1.7, 0.8
Mouse CD-1	320	^{134}Sr	0.5, 1.0, 1.9			
Mouse CBA/H				60	porous	0.8

* repetitive studies

man study using 4 μm monodisperse, ^{51}Cr labelled Teflon particles (Philipson et al., 1985).

Evaluation of Particle Transport Rate

Common to all species studied, long-term particle transport rate M(t) from the lungs to the GI-tract decreased drastically with time. In those studies where M(t) was not explicitly tabulated or given as a function, M(t) was calculated according to equation 1 from the given retention function L(t) and the given estimate of S(t). In all species M(t) was approximated by a sum of two exponential terms:

$$M(t) = M_{01} \exp(-m_1 t) + M_{02} \exp(-m_2 t) \qquad M_0 = M_{01} + M_{02} \qquad (4)$$

In Table 2 the values of the parameters of M(t) and the number of subjects/animals studied are given for each particle material and each species / strain. It is emphasized that the evaluation of a second term clearly depended on the parameters of the first term and the period of observation, i.e. the entire period must have been long enough that the first term was negligible for a sufficient time to determine the second term. It is notable, however, that particle transport in dogs followed a single exponential term for almost 1000 days if the most rigid analysis of fecal particle excretion was applied. The measurements of Snipes et al. (1983) confirmed the monotonic decay of particle transport. But the function of M(t) they gave in the paper was one exponential term and a final constant rate. This approximation was used for all data sets obtained from three species and 4 particle sizes. In Table 3 data of the transport rate M(t) are given at various times t during the first 400 days after inhalation which show the effectiveness of the clearance mechanism of particle transport in the various species at those times.

The initial transport rate M_0=M(0) was extrapolated from data obtained during the first weeks after inhalation of the test particles. Fast particle clearance was not taken into account in these estimations. As indicated above the latter was predominantly ascribed to particle removal from the tracheobronchial tree. However, there was no means to determine at what time and to which extent clearance of particles deposited on the tracheobronchial tree had diminished. Therefore, the extrapolated value of M(0) might have represented a superposition of both the vanishing clearance from the tracheobronchial tree and the initial clearance from the alveolar region.

Species differences

Both the initial transport rate and the decay of the transport rate varied considerably between the species. In the upper panel of Figure 3 M(t) in man, baboon and dog is shown for both FAP and Co_3O_4 particles. In the lower panel M(t) is shown in guinea pigs, three strains of rats, hamster and mice. Generally, in man and large animals the initial transport rate M_0 was in the range of 0.001 d^{-1} of the contemporary lung content. This was about an order of magnitude less than in mice, hamsters and the various strains of rats, where M_0 was $\geq$0.01 d^{-1}. It was quite astonishing that for each species of the rodents M_0 was very similar even though different strains had been studied. The latter was most striking in the three strains of rats studied. Interestingly, M_0 in guinea pigs was closer to man and large animals than to the other rodents. M_0 in man and baboon was very similar but in dogs it was even less according to the most rigid data evaluation for ^{57}Co-FAP.

The rate at which the particles reached the distal end of the mucociliary escalator of the tracheo-bronchial tree depended on the velocity of migration of the laden macrophages and the distance they had to travel. As was reviewed recently (Oberdörster, 1988) the velocity was effected by chemotactic and other biochemical factors eventually resulting in different velocities in different species. Interestingly, there were fewer airway

Table 2.

Parameters of the exponential terms of the particle transport rate M(t) according to equation 4 for man and various experimental animal species and for two different particle materials, FAP and Co_3O_4. Additionally, human data are given for Teflon particles. The period of observation is also given.

Species	Particle	Days of Observ.	M_{01}	m_1	M_{02}	m_2
Man	^{85}Sr-FAP/					
	^{88}Yt-FAP	400	.0031	.013	.00031	.0001
	$^{57}Co_3O_4$	700	.0018	.0059	.00040	.0016
	^{51}Cr-Teflon	300	.0090	.026	.00041	<.0001
Baboon	FAP		--	--		
	$^{57}Co_3O_4$	200	.0017	.013		
Dog	^{57}Co-FAP	1000	.0006	.0042		
	^{134}Cs-FAP	800	.005	.03	.0001	<.0001
	$^{57}Co_3O_4$	800	.0019	.067	.0004	.0039
Guinea pig	^{134}Cs-FAP	200	.005	.03	.0001	<.0001
	$^{57}Co_3O_4$	400		.0036	.0016	
HMT-rat	^{85}Sr-FAP	400	.019	.018	.0045	.0039
	$^{57}Co_3O_4$	400	.019	.028	.0066	.0056
F-344-rat	^{134}Cs-FAP	200	.020	.007	.001	<.0001
	$^{57}Co_3O_4$	200	.024	.009		
Long-Evans	FAP		--	--		
rat	$^{57}Co_3O_4$	200	.021	.019		
Hamster	^{85}Sr-FAP	500	.010	.014	.004	.0031
	$^{57}Co_3O_4$	400	.0022	.033	.0082	.0031
Mouse	^{134}Cs-FAP	200	.02	.006	.0015	<.0001
	$^{57}Co_3O_4$	300	.018	.0042		

generations in the zone of alveoli to respiratory bronchioli in rodents than in dogs, monkeys and man (Phalen et al., 1983), i.e. the distance from an alveolus to the terminal bronchiolus is shorter. This structural difference might have contributed to the larger initial transport rates M_0 and the subsequent, more effective transport in rodents compared to man and the large animals.

The decay of particle transport was even more variable between the various species than its initial value (Figure 3). In man and dog M(t) decreased in parallel but in baboons it vanished faster. In the latter species, the period of observation was to short to deter-

Table 3.

Data of the particle transport rate M(t) at various times t for man and various experimental animal species and for two different particle materials, FAP and Co_3O_4. Additionally, human data are given for Teflon particles. The period of observation is also given.

Species	Particle	Days of Observ.	M(0)	M(100)	M(200)	M(400)
Man	^{85}Sr-FAP/					
	^{88}Yt-FAP	400	.0034	.0012	.00053	.00031
	$^{57}Co_3O_4$	200	.0022	.0014	.00086	.00034
	^{51}Cr-Teflon	300	.0094	.0011	.00048	.00041
Baboon	FAP		--	--		
	$^{57}Co_3O_4$	200	.0017	.00046	.00013	
Dog	^{57}Co-FAP	1000	.0006	.00039	.00026	.00011
	^{134}Cs-FAP	800	.0051	.00035	.00011	.00010
	$^{57}Co_3O_4$	800	.0023	.00027	.00018	.00008
Guinea pig	^{134}Cs-FAP	200	.0051	.00035	.00011	
	$^{57}Co_3O_4$	400	.0036	.0031	.0026	.0019
HMT-rat	^{85}Sr-FAP	400	.024	.0062	.0026	.00096
	$^{57}Co_3O_4$	400	.026	.0049	.0022	.00070
F-344-rat	^{134}Cs-FAP	200	.021	.0109	.0059	.0022
	$^{57}Co_3O_4$	200	.024	.0098	.0040	
Long-Evans rat	FAP		--	--		
	$^{57}Co_3O_4$	200	.021	.0031	.00047	
Hamster	^{85}Sr-FAP	500	.014	.0054	.0028	.0012
	$^{57}Co_3O_4$	400	.010	.0061	.0044	.0024
Mouse	^{134}Cs-FAP	200	.022	.013	.0075	.0033
	$^{57}Co_3O_4$	300	.018	.012	.0078	

mine a second term of M(t) (equation 4) indicating a slower decay or a final, more constant transport rate. The longest studies including excretion analysis up to 700 days in man (Foster et al. 1989; Pearman et al., 1990) and 850 days in dogs (Kreyling et al. 1988) suggested that there was no final constant transport rate but M(t) continued to decrease.

In guinea pigs, different decays of M(t) were observed for the two particle materials of FAP and Co_3O_4. In the 250 days study, M(t) of FAP decreased parallel to that of dogs to a final constant rate of 0.0001 d^{-1}, while M(t) of Co_3O_4 remained almost constant above 0.001 d^{-1} throughout 360 days, resulting in a more efficient particle transport during time.

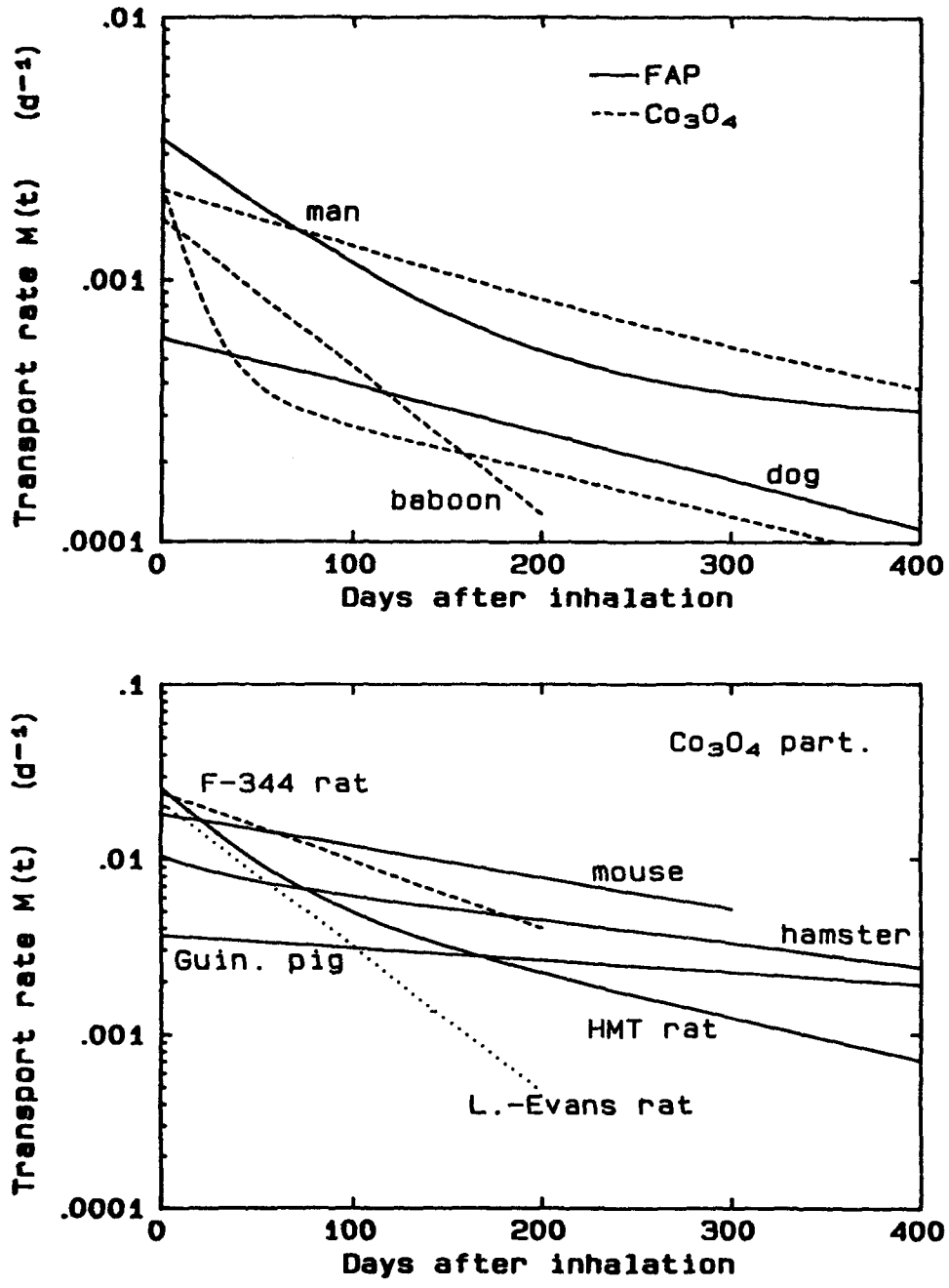

Figure 3. Particle transport M(t) of FAP and Co_3O_4 particles in man, baboon and dog (upper panel). Particle transport M(t) of Co_3O_4 particles in guinea pigs, three strains of rats (F-344 rat, HMT rat, Long-Evans rat), hamster and mice (lower panel).

Unfortunately in the FAP study no excretion data were given but only the function of M(t) which was derived from an entire lung clearance model (McClellan et al., 1984). The differences of M(t) observed in the two studies could reflect material dependence of particle transport but also differences between the two strains of guinea pigs.

Differences were observed between F-344 rats and the two other strains of rats independent of the particle material (Figure 3, lower panel). While M(t) diminished quickly in

HMT-rats and Long-Evans rats with a half-life of 40 days or less, the transport rate decreased more slowly in F-344 rats with 100 days half-life, i.e. particle transport in F-344 rats remained more effective than in the other two strains of rats. Unfortunately, M(t) was not determined in Sprague-Dawley rats, but lung retention decreased similar as in HMT rats and Long-Evans rats suggesting a similar, rapidly decreasing M(t) in these three strains. Hamsters and mice showed an even slower decrease of M(t) than F-344 rats (Figure 3, lower panel) with a half-life of 200 d and 140 d, respectively. Interestingly, no differences in the slow decrease of M(t) were found in the two strains of mice.

Particle transport to the hilar lymph nodes (TBLN) (Snipes et al., 1983; McClellan et al. 1984; Kreyling et al. 1986, 1988, 1989a; Harmsen et al., 1985) and observations of particles retained in the interstitium and subpleural spaces emphasized penetration of the particles through the epithelium. Significant fractions of particles had been found on the epithelium up to 500 days after inhalation by broncho-alveolar lavages (Kreyling et al. 1988, 1989a). From these findings, it is unclear how many of the particles remained on the epithelium. From those which penetrated through the membrane how many were carried to the TBLN? Also did particles penetrate reversibly through the epithelial barrier to appear again on the epithelium? In any event the decay of particle transport to the mucociliary escalator indicated an emptying pool of particles available for this transport mechanism which was macrophage mediated. Since the transport to TBLN was a minor and very slow mechanism, this means that an increasing fraction of particles was retained either on or beyond the epithelium in a state such that macrophages either could not phagocytize the particles or they phagocytized the particles but reached the mucociliary escalator increasingly more slowly.

Dependence on Particle Parameters

In each species except rats and guinea pigs, M(t) was independent of the two particle materials and the various particle sizes used, as shown in Table 3 by the data of M(t) at various time points. The three strains of rats showed clear differences in the decay of particle transport M(t). However, no material dependence of particle transport was found in F-344 rats and HMT rats in which both FAP or Co_3O_4 particles had been studied. In guinea pigs, different decays of M(t) were observed for the two particle materials of FAP and Co_3O_4 which might reflect differences in the two strains as discussed above. Interestingly, in man particle transport of another material, 4 μm ^{51}Cr labelled Teflon particles, was similar to those obtained for the other materials. Also no difference within a given species was found for porous and solid Co_3O_4 particles with a density of 2.5 - 3.5 and 6 g/cm3, respectively, (as long as M(t) was not too minute compared to S(t)). The particle size range varied in man from 1 to 4 μm geometric diameter (1.5 - 6 μm aerodynamic diameter) and in experimental animals from 0.5 to 2 μm (0.7 - 3.6 μm aerodynamic diameter). Administration of larger particles via inhalation would have been difficult since the particles would not have reached the peripheral lung due to their previous deposition in the upper airways.

Snipes et al. (1981,1984) found similar particle transport of 3 μm ^{141}Ce labelled polystyrene (PSL) particles in Fischer-344 rats and beagle dogs after administration via intratracheal instillation when compared to the inhalation data discussed here. However, in rats particle transport of 9 μm ^{85}Sr labelled PSL particles was much slower than that of the small PSL particles. Moreover, there was virtually no particle transport of 7.5 μm ^{85}Sr labelled PSL particles in dogs and no particle transport of 15 μm ^{46}Sc labelled PSL particles in either species during the entire four month period of observation. These results suggested that particle transport out of the peripheral lung of other species also might diminish for particles larger than 7-10 μm diameter. Since particle transport was macrophage mediated it is plausible to assume that the mobility of macrophages decreased with the increasing size of their load resulting in diminishing particle transport. The same ef-

fect of accumulated particle mass in macrophages had probably contributed to the vanishing test particle transport out of the lungs of various rodent species during chronic exposure of high concentrations of Diesel exhaust or other carbonaceous aerosols (Chan et al., 1984; Lee et al., 1987; Wolff et al., 1987; Muhle et al.; 1988, Bellmann et al., 1989).

Intersubject Variability and Reproducibility in Species

Intersubject variability of particle transport within a given species or strain was remarkably low for all rodents and dogs which were bred and maintained under controlled conditions of the various facilities. Moreover, the kinetics of particle transport in each of various species (F-344 rats, Syrian golden hamsters and the two strains of mice) were surprisingly reproducible in different investigations of different laboratories carried out more than three years apart from each other (Snipes et al., 1983; Collier et al., 1989; Talbot et al., 1989). Similarly, three studies on HMT rats at the National Radiological Protection Board, Chilton, UK, in 1985, 1986 and 1988 (Collier et al., 1989, submitted; Kreyling et al., 1989b) confirmed the invariance of the kinetics of particle transport within this strain. In an age-related investigation on the same species beginning at ages of 3, 13, 21 and 46 weeks Collier et al. (submitted) found no significant changes in particle transport M(t). As mentioned above the differences of M(t) found in guinea pigs for FAP and Co_3O_4 particles might be associated with the different strains.

Due to the predominant contribution of translocation S(t) to the clearance of porous Co_3O_4 particles in beagle dogs, M(t) could only be evaluated satisfactorily from studies using solid Co_3O_4 particles. Therefore, M(t) was obtained from only four animals, but intersubject variation was very low and matched excellently with the data for ^{57}Co-FAP for which the most rigid analysis was applied (Kreyling et al., 1988). These data are in good agreement with those obtained from 120 beagle dogs (Snipes et al., 1983). In baboons, wild-caught as young animals in West Africa, the intersubject variation of the kinetics of particle transport was slightly larger than in the other species discussed above. Yet, the mean pattern of the four animals studied in 1985 (André et al., 1989) was similar to those of the two animals studied in 1988 (Kreyling et al., 1989b). Largest intersubject variability was observed in man in each of the three studies (Bailey et al., 1985b; Philipson et al., 1985; Foster et al., 1989).

ESTIMATED LUNG CONTENT DURING CHRONIC EXPOSURE

Particle transport M(t) of the test particles was determined while the human volunteers and the experimental animals were continuously exposed to the ambient aerosol which contained a certain fraction of nearly insoluble particles. Hence, M(t) was determined during chronic exposure of the insoluble particle fraction of the ambient aerosol. Using the functions of M(t) in Table 2 obtained from the interspecies comparisons, the accumulated lung burden in each species was estimated during chronic exposure to an aerosol of constant concentration. It was assumed that (1) in each species a unit dose D_o of particle mass was deposited per day and (2) this material was exclusively cleared by particle transport, i.e. translocation S(t) = 0 during the entire period in all species. According to equation 1, the rate of change of retention becomes the result of the daily intake D_o and clearance M(t) of the lung retention L(t):

$$\frac{dL(t)}{dt} = \{ D_o - M(t) \}\ L(t) \qquad (5)$$

If $M(t) = M_0$ is constant, lung retention L(t) is:

$$L(t) = \frac{D_0}{M_0} \{1 - \exp(-M_0 t)\} \quad (6)$$

where $L(\infty) = D_0/M_0$ is the limiting value to which lung retention approximated during time. This value in units of the deposited dose D_0 and the time t_{95} at which L(t) reached 95% of $L(\infty)$ are given in Table 4 for each of the species. The equilibrium values for man, baboon, dog and guinea pig were up to an order of magnitude larger than those of rodents. Similarly, in the same species the time interval was much longer to reach 95% the of the equilibrium value.

Table 4.

The approximate limit $L(\infty)$ of lung retention during chronic aerosol exposure when $M(t) = M_0$ is constant according to equation 6. $L(\infty)$ is given in units of the daily deposited dose D_0; t_{95} is the time when lung retention L(t) has reached 95% of the value of $L(\infty)$.

Species / strain	$L(\infty)$	t_{95} (days)
man	290	860
baboon	590	1740
dog	440	1280
guinea pig	280	810
HMT rat	40	100
F-344 rat	36	90
Long-Evans rat	48	120
Hamster	71	190
Mouse	47	120

Assuming M(t) was a function of time as given in Table 2, equation 5 was numerically solved and the accumulating lung retention L(t) in units of the daily deposited dose D_0 is shown in Figure 4 for the various species. No equilibrium value L(t) was reached in any of the species since M(t) was a decreasing function with time although the increase was very minute in F-344 rats and mice. Since particle transport was less effective in man, baboon and dog, the accumulative retained dose was much higher than in rodents. The smaller the values of M(t) were, the higher was the accumulated, retained lung burden. Additionally, the accumulated retained dose remained lower in those species or strains in which M(t) decayed more slowly with time such as F-344 rats and mice compared to HMT rats and Long-Evans rats.

Figure 4 shows that after 1000 days of exposure about 70% of the deposited dose has been retained in the human lungs while only 30% has been removed by particle trans-

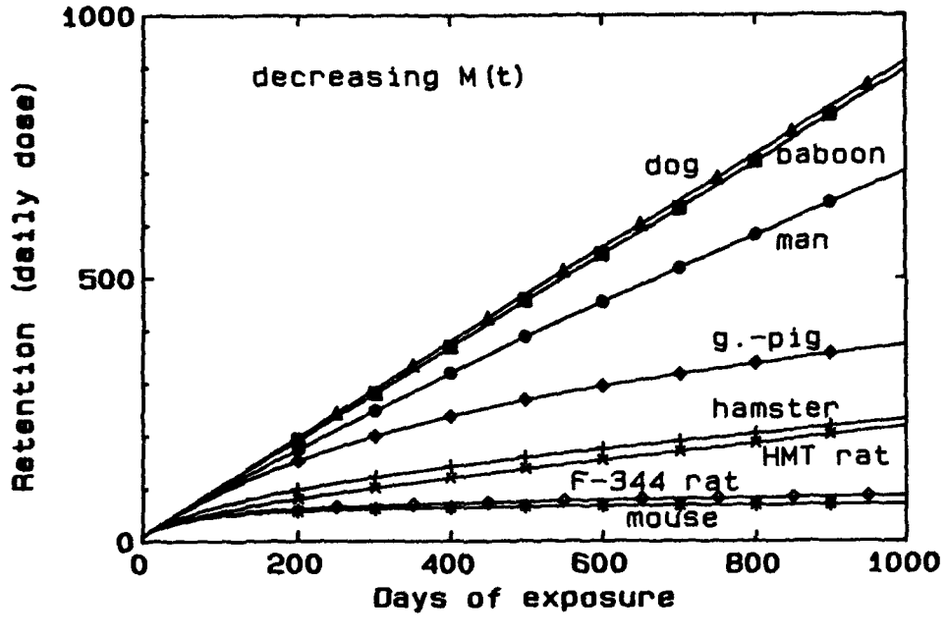

Figure 4. Estimates of the accumulated lung retention L(t) during chronic aerosol exposure to a daily deposited unity dose D_0.

port, whereas in F-344 rats and mice the accumulated retention would only account for about 10% of the deposited dose during a life time exposure. Applying this result to the chronic exposure of the ambient aerosol, it represents an estimated upper limit since particles are not completely insoluble and hence the translocation of dissolved material contributes to the total clearance of the particles from the lungs. The same is valid for other aerosols, for instance in specific occupational environments.

No limitations on the daily deposited dose were made in these calculations. It is well known if the deposited dose increases, recruitment of alveolar macrophages onto the epithelium also increases (Brain, 1985), eventually resulting in a more effective particle transport. However, the chronic exposure studies under "overload" conditions clearly indicate that beyond a certain administered dose, particle clearance even diminishes (Chan et al., 1984; Lee et al., 1987; Wolff et al., 1987; Muhle et al., 1988; Bellmann et al., 1989). Taking this and the less effective particle transport in man and large animal species into account, "overload" phenomena might occur at even lower concentrations during chronic aerosol exposure than was observed in rodents.

CONCLUSION

Particle transport from the alveolar epithelium to the beginning of the mucociliary escalator of the tracheo-bronchial tree was a slow, macrophage mediated clearance mechanism which decreased rapidly in all species studied. Both the initial transport rate M(0) and the kinetics of M(t) varied considerably between the species. As a result particle transport in man and large animal species was less effective by an order of magnitude than in rodents.

Particle transport was material independent in all species as long as the material was not cytotoxic to alveolar macrophages. It was also independent of the particle size within the range of 0.5 - 5 µm geometric diameter. Particle transport diminished for particles larger than 7 - 10 µm in rodents; but it is unknown in any species for fine and ultrafine

particles. Particle transport in rodents vanished during chronic aerosol exposure at high concentrations. The latter is unknown for man but it is plausible to assume a similar behavior. The estimated lung retention during chronic exposure which accumulated faster in man than in rodents suggested that "overloading" and decreasing particle transport might occur at even lower concentrations during chronic aerosol exposure than observed in rodents.

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Dust Overloading of Lungs: Investigations of Various Materials, Species Differences, and Irreversibility of Effects

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ABSTRACT

In separate inhalation investigations, rodents (Wistar rats, Fischer-344 rats, Syrian golden hamsters, NMRI and C57BL mice) were exposed to various dusts such as test toner (polymer pigmented with carbon black), polyvinyl chloride, carbon black, diesel exhaust and two crystalline forms of titanium dioxide (anatase and rutile). The animals inhaled various concentrations (0.8 to 64 mg/m^3) of these particles for up to 2 years.
Alveolar clearance retardation was detectable above a retained pulmonary burden of 0.5 mg per rat lung, and a substantial decrease in the clearance rate (about a factor of 6) was observed following heavy dust loading, exceeding 10 mg dust per rat lung. Above a threshold lung burden, signs of lung overloading persisted 15 months after cessation of exposure in F-344 rats. Retardation of alveolar clearance was also observed in hamsters, commencing at higher lung burdens (normalized to lung weight) than in rats. At high dust exposure levels, persistent pulmonary inflammation was present in both species. In rats the concentration of lavagable cells remained constant, with decreased macrophages and increased polymorphonuclear neutrophils (PMN) noted, while in hamsters, the cell count increased substantially in both macrophages and PMN's. A retarded particle clearance was also observed in mice at a lung burden above 1 mg/lung.
These results, accompanied by published accounts, indicate that the lung overloading phenomenon is noted among a variety of species and materials. It is generally observed upon exceeding a threshold lung burden with particles of low solubility and low acute toxicity for considerable periods of time.

INTRODUCTION

The Fraunhofer Institute of Toxicology and Aerosol Research has investigated the inhalation toxicity of insoluble particles for more than 10 years. The studies were performed for various reasons; therefore different species, materials and exposure conditions were selected. Since several of these investigations were conducted under Good Laboratory Practice (GLP) guidelines according the Organisation for Economic Co-Operation and Development (OECD) , standard measurements such as body weight, food consumption, biochemical and

Key words: Lung, retention, alveolar clearance, insoluble particles, dust overloading, inflammation, fibrosis

hematologic parameters, organ weight, histopathologic examinations etc. were performed. In addition, in most of these investigations we measured particle deposition, clearance and retention, a number of biochemical and cytological parameters and pulmonary function.
The characteristic findings at the highest exposure level in most of these rodent inhalation studies included: increased lung weight, disproportionate retention of test material, i.e. the ratio of the retained mass to the aerosol concentration increased, decreased or obliterated alveolar clearance, impaired pulmonary function and persistent inflammatory responses. These changes were reflected by a variety of histopathological alterations which are only partly covered within this paper and are reported separately. The pulmonary changes were ascribed to "lung overloading", a generic response of the lung to the long-term presence of large quantities of insoluble particles. Dust overloading of the lungs was reported in various studies (Ferin and Feldstein 1979; Vostal et al. 1982; Muhle et al. 1984 and 1988; Vincent et al. 1985; Wolff et al. 1987). This paper will focus on some of the common outcome characteristics of various studies conducted in our laboratory.

MATERIALS AND METHODS

The properties of the various particles evaluated are shown in Table 1. The test toner used in copy machines was composed of about 90 % styrene/1-butylmethacrylate, a high molecular weight random copolymer (CAS 25213-39-2) and 10 % of a medium color, high purity furnace type carbon black (CAS 1336-86-4). A 9000-type xerographic toner material (Xerox Corp. Rochester, USA) was specially prepared for this study and was enriched about 10-fold in respirable sized particles, relative to the commercial toner, such that it was about 35 % respirable according to the American Conference of Governmental Industrial Hygienists (ACGIH) criteria.
Polyvinyl chloride powder (PVC, Chem. Werke Hüls, FRG) and two types of titanium dioxide (TiO_2, rutile, type "Bayer Titan T", Bayer AG, FRG and anatase, type "P 25", Degussa, FRG) were used. A characteristic difference between the two types of TiO_2 was the diameter of the primary particles (Table 1).

TABLE 1

Properties of the Particles

Particle type	Diameter of primary particles [µm]	Mass median aerodynamic diameter [µm]	Geometric standard deviation	Density [g/cm³]
Test toner (carbon black pigmented polymer)	~4	4	1.5	1.15
Polyvinyl chloride powder(PVC)	~1.3	1.3	2.07	1.3
Titanium dioxide (rutile)	0.2-0.7	1.1	1.6	4.26
Titanium dioxide (anatase)	0.02-0.04	0.80	1.8	3.84
Carbon black (Printex 90)	~ 0.014	0.64	2.1	~ 2
Diesel exhaust particles	~ 0.05	0.25	2.9	~2

High purity carbon black (type "Printex 90", Degussa, FRG) was taken as a reference material to diesel exhaust. The fraction extractable by toluene was < 0.1 %.

A 40 kilowatt 1.6 l diesel engine served as a source for the exhaust emissions. The engine was mounted on a computer-controlled test bench and was operated continuously according to the US 72 test driving cycle. For further details see Heinrich et al. (1986).

A dry aerosol dispersion technique was used for the first 5 types of particles listed in Table 1 (Koch et al. 1986). Aerosols of carbon black and anatase consisted of aggregates of primary particles.

Animals were exposed in horizontal flow type whole body inhalation chambers (Heinrich et al. 1985) at aerosol concentrations listed in Tables 2 and 3. Animals inhaled one of these test materials for up to two years. Results shown in these tables originate from female rats; pooled data of males and females are given only in Study A. In Study E, Syrian golden hamsters were used. After serial sacrifices during the course of the studies, the retained mass in lungs was analyzed (5-8 lungs per investigation). After digestion of the lung tissue, toner, PVC, carbon black and diesel soot were determined by light absorption spectroscopy. Titanium dioxide was analyzed by atomic absorption spectroscopy.

Alveolar clearance was determined at various times within the studies using short-term nose only exposure to radioactive labelled tracer particles (^{85}Sr-polystyrene with an MMAD of about 3.5 µm). The decrease of the γ-activity in the thoracic area was measured over a 75-100 days period (Bellmann et al. 1989) .

For calculation of the clearance rate coefficients or half-times, an exponential curve fit was performed on these data excluding measurements before day 15 to omit faster clearance processes of the upper respiratory tract and to allow a simplified and concise description of the clearance kinetics.

The total dust concentration and the duration of exposure of the various studies, the retained mass, the retained volume and the half-time of the alveolar clearance are presented in Tables 2 and 3. With the exception of Study A the retained mass reflects the value at the middle of the period in which clearance of labelled particles was measured, which enables documentation of the relationship between both parameters. In cases in which the retention was not determined at this date, the values were interpolated from adjacent measurements.

Bronchoalveolar lavagate was obtained by a twofold lavage with 4 ml saline. The lavagate was analyzed for cytological and biochemical parameters (Henderson et al. 1987).

For statistical analysis, data were examined by analysis of variance (ANOVA), followed by Dunnett's test to compare various treatment groups with controls.

RESULTS

Subchronic Inhalation Study of Toner in Rats (study A)

Fischer-344 rats were exposed for 90 days to 1, 4, 16 and 64 mg/m^3 of toner. The retained quantities of toner at the end of the exposure period are shown in Table 2. The greater than proportional increase of retained test material at 16 and 64 mg/m^3 compared to 4 mg/m^3 can be seen in Figure 1, where retention is normalized by the product of aerosol exposure concentration and minute volume. The minute volume correction was applied for pooling the results for male and female animals, which had slightly different minute volumes. The latter was calculated from the body weight using the procedure of Stahl (1967). In this investigation, alveolar toner clearance was determined using the retained quantity of toner in excised rat lungs. The results of both sexes are combined only in this study because this direct determination of the retention kinetics led to higher statistical fluctuations compared to the measurements with labelled particles which were used in the other studies. Animals were sacrificed at days 1, 25, 50 and 75, after removal from exposure. Retention

TABLE 2

Mean and Standard Deviation of Exposure Atmospheres, Retained Mass and Particle Volume in Lungs and Half-times of Alveolar Clearance (with 95% Confidence Limits) in Four Different Inhalation Studies in Rats

Material	Total dust concentration [mg/m³]	Retained mass [mg/lung]	Retained volume [µl/lung]	Half-time of alveolar clearance [days]	
				^{85}Sr PS Mean (95% CL)	Test material[a] Mean (95% CL)
Study A. Exposure: 30 hr/week for 3 months (Muhle et al., 1990a)					
Toner	1.0 ± 0.2	0.085 ± 0.032	0.074		79 (34-∞)
Toner	4.0 ± 0.6	0.275 ± 0.059	0.239		86 (52-245)
Toner	16.1 ± 1.4	1.86 ± 0.28	1.62		186 (107-636)
Toner	63.2 ± 5.3	11.5 ± 0.8	10.0		>1000 (276-∞)
Study B. Exposure: 30 hr/week for 22.5 months (Muhle et al., 1990b, Bellmann et al. 1990)					
Control air	0	0	0	65 (57-77)	
Toner	1.0 ± 0.1	0.19 ± 0.04	0.169	* 84 (74-98)	
Toner	4.1 ± 0.1	1.36 ± 0.36	1.18	** 187 (122-402)	
Toner	16.0 ± 0.9	12.2 ± 1.3	10.6	** 307 (150-∞)	
TiO_2 (Rutile)	5.0 ± 0.7	2.21 ± 0.37	0.519	** 93 (78-117)	
Study C. Exposure: 25 hr/week for 8 months (Bellmann et al., 1986)					
Control air	0	0	0	57 (51-63)	
PVC	3.3 ± 0.5	0.56 ± 0.16	0.43	71 (64-80)	85 (65-123)
PVC	8.3 ± 0.9	2.09 ± 0.29	1.61	** 122 (95-164)	120 (92-169)
PVC	20.2 ± 1.8	7.24 ± 1.10	5.57	** 184 (108-630)	277 (164-884)
Study D. Exposure: 95 hr/week for 4.5 months (Creutzenberg et al., 1990)					
Control air	0	0	0	61 (52-76)	
Diesel exhaust	0.8 ± 0.5	0.95 ± 0.25	0.47	** 109 (72-224)	
Diesel exhaust	2.4 ± 1.2	4.67 ± 1.19	2.3	** 292 (211-475)	
Diesel exhaust	6.8 ± 1.6	14.4 ± 2.3	7.2	**>1000 (902-∞)	
Carbon black	7.4 ± 1.5 [b]	13.7 ± 2.0	6.9	** 472 (283-1420)	
TiO_2 (Anatase)	7.2 ± 1.2 [b]	14.2 ± 2.2	3.7	**>1000 (548-∞)	

- Levels of significance (Dunnett's test of corresponding clearance rate coefficients)
  * $P < 0.05$ ** $P < 0.01$

- [a] Clearance measurement of toner or PVC particles after cessation of exposure

- [b] Exposure concentrations during the first 4 months:
  7.4 ± 1.5 and 7.2 ± 1.2 mg/m³ for carbon black and TiO_2, respectively.
  Corresponding values for the 4 following months: 12.0 ± 1.5 and 14.8 ± 3.2 mg/m³
  and after 8 months 12.3 ± 1.9 and 9.4 ± 3.2 mg/m³, respectively.

TABLE 3

Mean and Standard Deviation of Exposure Atmospheres, Retained Mass and Particle Volume in Lungs and Half-times of Alveolar Clearance (with 95% Confidence Limits) in Male Hamster in Study (E)

Material	Total dust concentration [mg/m³]	Retained mass [mg/lung]	Retained volume [µl/lung]	Half-time of alveolar clearance (^{85}Sr PS) Mean (95% CL) [days]
Study E. Exposure: 30 hr/week for 10.5 months				
Control air	0	0	0	109 (91-135)
Toner	4.0 ± 0.3 *	0.11 ± 0.03	0.10	* 72 (56-102)
Toner	16.0 ± 0.3 *	0.43 ± 0.06	0.37	103 (86-127)
Toner	63.7 ± 1.9 *	3.03 ± 0.66	2.63	** 246 (150-672)
TiO_2 (Rutile)	30.6 ± 2.1 *	16.7 ± 1.24	3.88	** 641 (279-∞)

- Levels of significance (Dunnett's test of corresponding clearance rate coefficients)
  * $P < 0.05$ ** $P < 0.01$

- * Exposure concentrations during the first 5 months:
  1.5 ± 0.2, 6.1 ± 0.4, 24.5 ± 1.6, 39.3 ± 7.3 mg/m³

data of days 25-75 were fitted by an exponential curve $M = M_0 e^{-kt}$. The clearance rate coefficient k with the standard error range was obtained for each group by linear regression.
The calculated alveolar clearance half-times are shown in Table 2. At the aerosol concentrations of 1 and 4 mg/m^3, appreciable test material clearance, with a retention half-time around 80 days, was obtained. Some retardation of clearance was observed at 16 mg/m^3.

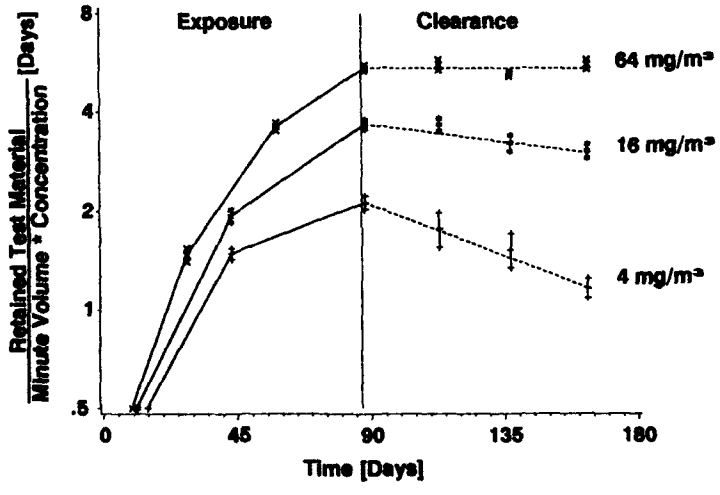

FIGURE 1. Lung Burden of Test Toner during the 90-Day Inhalation Study of Toner in Rats and 90-Day Post-Exposure Period, Normalized to the Minute Volume of the Rats and Exposure Concentration. Pooled Data for Male and Female Animals.

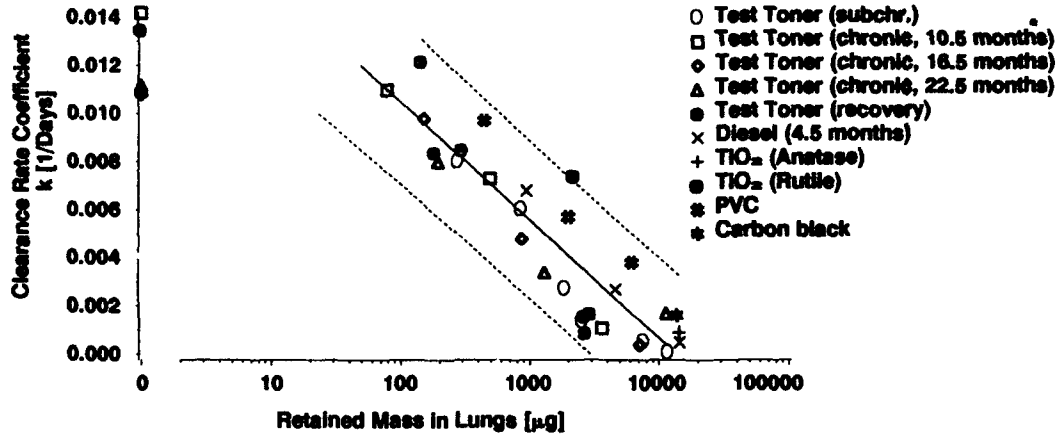

FIGURE 2. Clearance Rate Coefficient of Labelled Particles (^{85}Sr-Polystyrene) or Toner Particles as a Function of the Retained Mass of the Various Test Materials. Compilation of the Various Studies performed in Rats. Lines: Regression Curve with 95% Confidence Interval

At the highest exposure concentration (64 mg/m^3), toner particle clearance had practically ceased. An illustration of the various clearance rates at the respective pulmonary loads (and exposure concentrations) is shown in Figure 2, indicated as test toner (subchr.).
For comparison of effects of particles differing in density, the retained volume of particles is a more useful parameter than the retained mass, as alveolar macrophages show an upper volumetric uptake limit (Bowden 1987, Morrow 1988). Results presented in Figure 3 demonstrate the decrease of the clearance rate coefficient as a function of the retained dust volume in the lungs. This graph also contains results of further studies which will be introduced later. For further details of this study see Muhle et al. (1990a).

Chronic Inhalation Study of Toner and TiO_2 (Rutile) in Rats (Study B)

The quantity of test toner and TiO_2 retained in the lungs of female Fischer-344 rats at 22.5 months of exposure is summarized in Table 2, Study B.

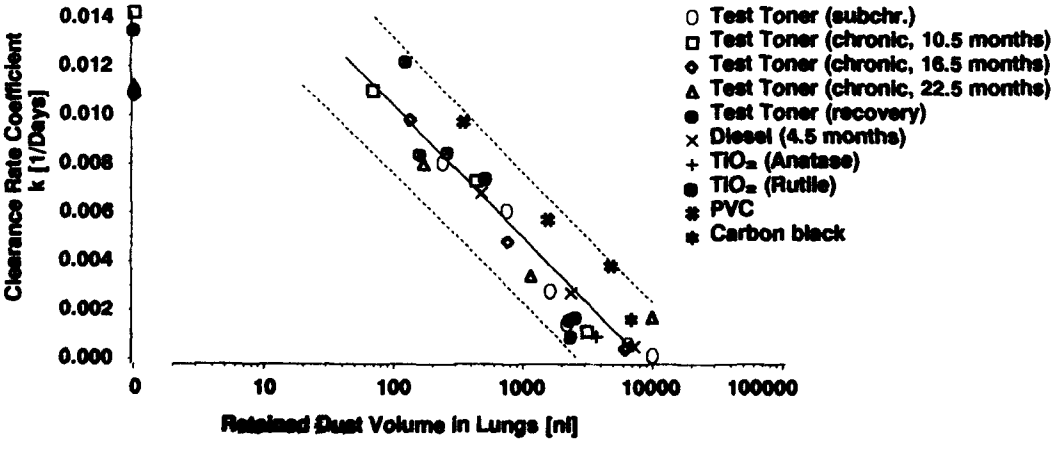

FIGURE 3. Clearance Rate Coefficient of Labelled Particles (^{85}Sr-Polystyrene) or Toner Particles as a Function of the Retained Dust Volume of the Various Test Materials. Compilation of the Various Studies performed in Rats. Lines: Regression Curve with 95% Confidence Interval

The lung burden in males was about 40 % higher than in females, which is influenced by the higher body weight and minute volume of the males. For better comparison with other rat studies, only the data of females are shown in Table 2, Studies B to D. The lung weight in the toner high exposure group was increased by 40 % at 22.5 months of the study.

In Table 2, the clearance half-time values with 95 % confidence limit for the tracer aerosols are presented. Half-times were 65 days for controls. Retardation of alveolar clearance started when the retained mass in lungs reached a level of about 0.5 mg per rat lung and amounted to about 300 days after heavy dust overloading (>10 mg per rat lung). This is also documented by Figure 2 in which these results are shown under test toner (chronic). Also included in this figure are clearance and retention data after 10.5 and 16.5 months of the study, which are not listed in Table 2.

Figure 4 shows the retained material in lungs normalized to the aerosol concentration. In the absence of lung overloading, the three lines for toner should be superimposable. This Figure demonstrates the overproportional increase of the toner lung burden in the 4 and 16 mg/m^3 exposure groups compared to the 1 mg/m^3 exposure group. This illustrates the slight overloading of lung clearance in the medium and substantial overloading in the high toner exposure groups.

A semi-empirical model was developed for calculation of retained masses in rat lungs. This model takes into account the relationship between the retained mass (m) and clearance rate coefficient (k) as documented by Figure 2. Further, the deposited mass per time (D) can be calculated from the deposition fraction, the aerosol concentration and the inhaled volume per time. The time dependence of the retained lung burden m is described by the relationship

$$dm/dt = D - km$$

Through an iterative process the lung burden at day (t+1) can be calculated from the previous day's lung burden (m_t).

$$m_{(t+1)} = D + (1-k)\, m_{(t)}$$

The lines in Figure 4 are calculated by this model.

An exposure-related decrease in the fraction of lavagable macrophages and an increase in the fraction of lavagable polymorphonuclear neutrophils (PMN) and

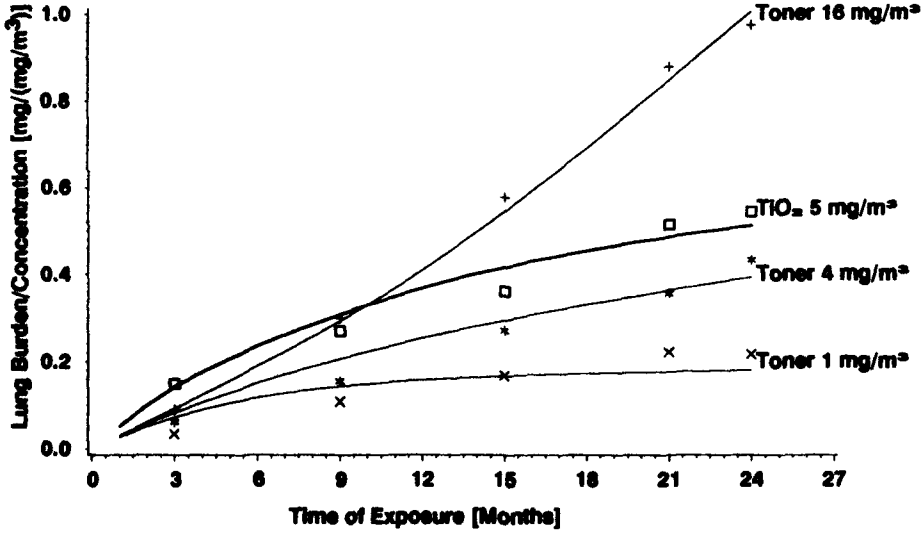

FIGURE 4. Lung Burden of Test Materials Pooled for Male and Female Rats, Normalized to the Exposure Concentration. Chronic Inhalation Study of Toner and TiO_2 (Study B). Lines: Model Calculation

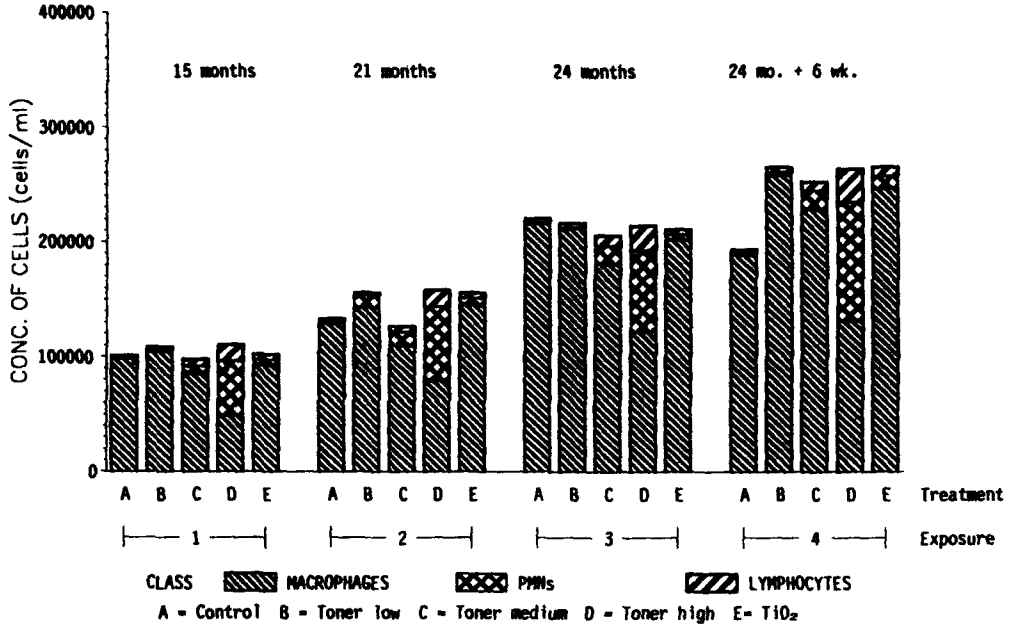

FIGURE 5. Differential Cell Count in Bronchoalveolar Lavagate up to 24 Months of Exposure to Toner and TiO_2 in Rats. Aerosol Concentration as Listed in Table 2, Study B.

lymphocytes were observed at the toner middle and high exposure level in the bronchoalveolar lavagate, indicating persistent inflammatory responses. This cytologic pattern persisted throughout the study as the results at 15, 21, 24 and 25.5 months were quite similar (see Figure 5). The results from the toner low and TiO_2-exposed groups were comparable to control.
An important lesion observed was lung fibrosis, the extent of which was found to increase with both toner exposure level and duration. In those animals which showed a survival time of more than 21 months, a slight to moderate degree of fibrosis in 92 % of the rats at the toner high and a very slight degree of fibrosis in 22 % at the toner middle exposure were observed. There was no fibrosis reported in the toner low exposure group at any time. Detailed data are presented by Muhle et al. (1990b) and Bellmann et al. (1990).

Recovery Study after Subchronic Inhalation of Toner in Rats

The reversibility or permanence of lung overloading was investigated in a follow-up study using Fischer-344 rats and the same test toner.
Exposure conditions were selected to achieve dust overloading conditions in the high exposure group (40 mg/m^3) but not in the low exposure group (10 mg/m^3) after 3 months of exposure. A 15-month post-exposure period followed with periodic measurements.
The quantity of test toner retained in the lungs was determined at 3, 6, 9, 12 and 18 months of the study. The values of retained masses after 3 months of exposure were 0.4 and 3.0 mg/lung at the low and high toner exposure levels, respectively (see Table 4). The corresponding values after 15 months of clearance were 0.12 and 2.6 mg/lung, respectively.
The alveolar toner clearance half-times were calculated from the toner retention measurements during the post-exposure period. For the high dose group a half-time of about 2800 days and for the low dose group a half-time of 277 days were calculated. If one assumes that the quantities of toner present at 15 months post exposure are sequestered in the lungs into compartments without clearance, the corresponding half-times after subtraction of this amount are 51 and 321 days, respectively.

TABLE 4

Retention of Toner in Lungs after Three Months of Exposure and a Subsequent Observation Period of 15 Months.

Total dust concentration [mg/m³]	Retained mass [mg/lung]					Calculated clearance half-time of toner [days]	
	Exposure + observation period [months]						
	3	3 + 3	3 + 6	3 + 9	3 + 15		Assuming sequestration
10.1±0.2	0.40±0.05	0.20±0.06	0.16±0.04	0.12±0.03	0.12±0.05	277	51
40.9±1.3	3.01±0.40	2.86±0.21	2.51±0.35	2.77±0.26	2.65±0.33	2845	321

Labelled particles, polystyrene latex (^{85}Sr, MMAD-3.5 µm) were periodically inhaled by the nose-only route and were used to measure alveolar clearance. The alveolar clearance of the tracer aerosol was retarded at both toner exposure levels (Table 5). At the low exposure level, the degree of clearance retardation was slight and a partial recovery of the clearance behavior was noted after six months post-exposure. In contrast, at the toner high exposure level, alveolar clearance was substantially impaired and no indication of a reversal in this response was apparent during the 15-month observation period.

TABLE 5

Alveolar Clearance Half-time of Labelled Particles after Three Months of Exposure to Toner and a Subsequent Observation Period of 15 Months

Group Dust concentration [mg/m³]	Alveolar clearance half-time with 95% confidence interval [days]			
	Exposure + observation period [months]			
	3	3 + 3	3 + 6	3 + 12
	Mean (C.L.)	Mean (C.L.)	Mean (C.L.)	Mean (C.L.)
Control	45 (39-54)	45 (42-49)	39 (35-45)	75 (58-105)
Toner 10	66 (55-84)	81 (71-96)	55 (49-61)	86 (68-117)
Toner 40	229 (170-352)	635 (378-1991)	329 (217-678)	308 (209-579)

Bronchoalveolar lavage was performed periodically. The results of the differential cell count are presented in Figure 6. A large influx of PMNs and a moderate increase in lymphocytes as well as an increase in lavaged cell concentration were observed in the toner high group. The increase in elevated PMN and lymphocyte responses persisted throughout the observation period of 15 months.

Histopathological investigations after the 90-day exposure period showed a multifocal intra-alveolar accumulation of particle-laden macrophages to a moderate degree in the 40 mg/m^3 exposure group and minimal changes in the 10 mg/m^3 group. No fibrosis was diagnosed at this stage. After a 15-month observation period, a treatment-related interstitial fibrosis of a mild focal nature was observed in the subpleural region in two out of five rats in the toner high exposure group, with no fibrosis in the low exposure group. This delayed appearance of fibrosis is interpreted as a result of the persistent inflammatory reaction in the lungs and is a consequence of dust overloading. For further results see Bellmann et al. (1989) and Creutzenberg et al. (1989).

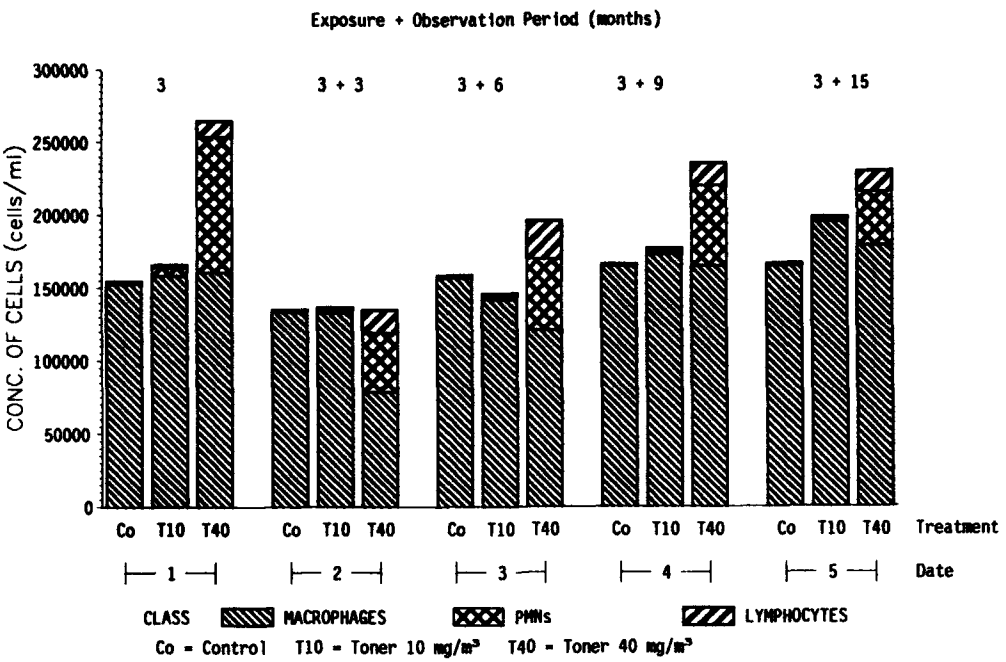

FIGURE 6. Differential Cell Count in Bronchoalveolar Lavagate Showing Persistence of PMN Presence During a 15-Month Recovery Period.

Inhalation Study with Polyviny Chloride (PVC) Powder in Fischer-344 Rats (Study C)

The exposure conditions and the retained mass after a seven-month exposure period are listed in Table 2, Study C. The retention of the inhaled PVC powder was determined at 1, 3 and 5 months after cessation of exposure. After exposure to high PVC concentrations the alveolar clearance of the inhaled material was slower by a factor of 3.3 compared to the exposure to the low concentration. Similar effects were found for the clearance of a superimposed spike of ^{85}Sr-polystyrene which showed a retardation factor of 3.2, compared to the control group (see Table 2). At the medium PVC exposure concentration, there was a moderate or slight retardation of lung clearance. Obviously the ^{85}Sr-polystyrene particles served as good surrogate particles to follow alveolar clearance. A dose-dependent increase of PMN was detected in the bronchoalveolar lavage after 8 months inhalation of PVC powder.

Chronic Inhalation Study of Toner and TiO_2 (Rutile) in Hamsters (Study E)

The aerosol concentrations in this study were changed after 5 months as indicated in the footnote of Table 3. For the three toner groups the aerosol concentration was increased by a factor of 2.7 whereas for TiO_2 the concentration was lowered by one quarter. The reason for this change was that interim retention measurements had shown that the desired similar volumetric lung burden in the toner high and TiO_2 exposure group would not have been reached by the original values.
The quantity of test toner and TiO_2 retained in the lungs of male hamsters at 16.5 months of exposure is shown in Table 3.
The lung weight was increased by 36 % and 77 % in the toner high and TiO_2 exposure groups, respectively, at 18 months in the study. The retained mass in lungs normalized by the aerosol concentration is shown in Figure 7. The graph shows an overproportional increase of the lung burden in the toner high exposure group.
The alveolar clearance half-times for male hamsters after an exposure period of 10.5 months are shown in Table 3, documenting the phenomenon of "dust

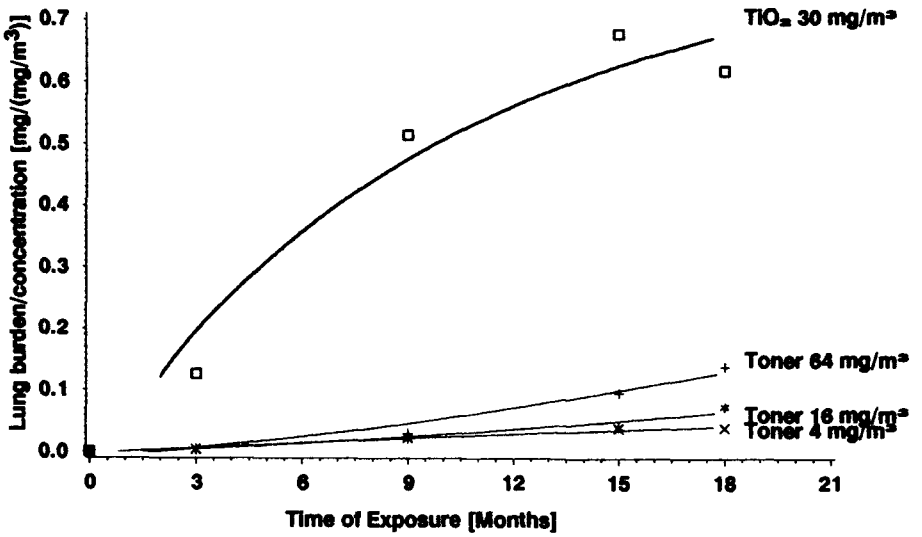

FIGURE 7. Lung Burden of Test Materials for Male Syrian Golden Hamsters, Normalized to the Exposure Concentration. Chronic Inhalation Study of Toner and TiO_2 (Study E)

overloading" also in hamsters. The corresponding values of the clearance rate coefficent are presented in Figure 8. Unlike the results in rat studies, a slight amount of retained dust (0.01 to 0.5 mg per hamster lung) seemed to accelerate the alveolar clearance. It appears that the critical lung burden which leads to a retardation of particle clearance is reached earlier in male than in female hamsters. This interpretation is in accordance with the lower amount of retained toner in the lung of female hamsters. This may be

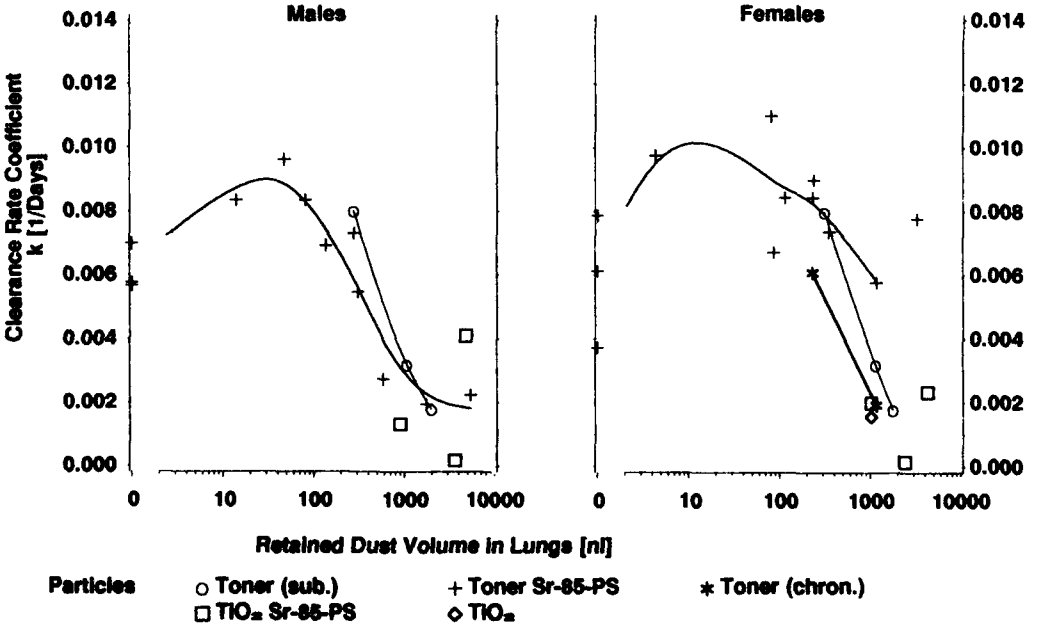

FIGURE 8. Clearance Rate Coefficient of Labelled Particles (^{85}Sr-Polystyrene) or Toner Particles as a Function of the Retained Dust Volume of Toner and TiO_2. Subchronic and Chronic Inhalation Studies in Syrian Golden Hamsters.

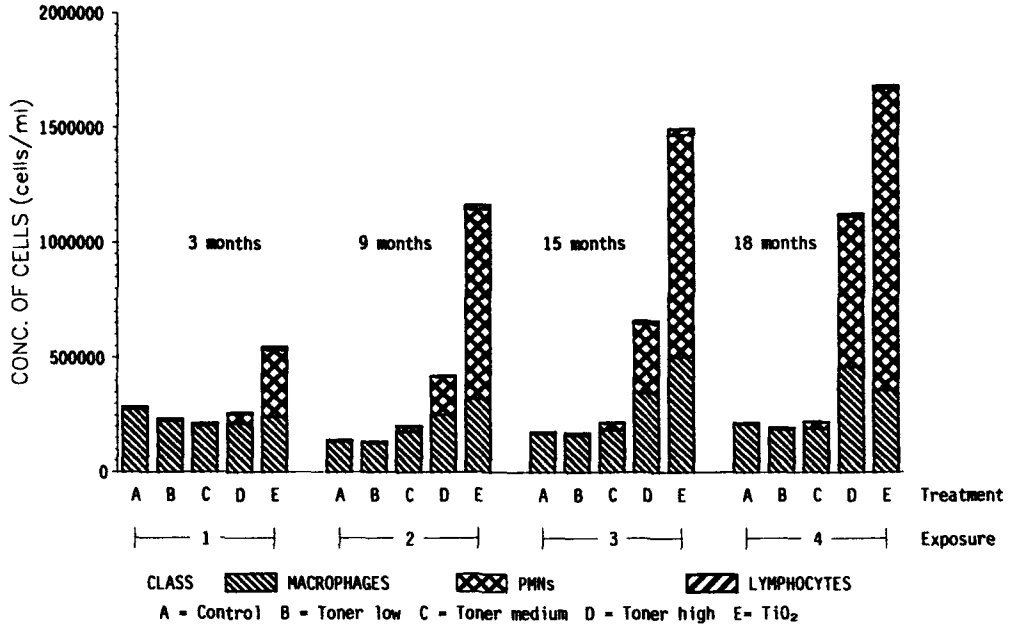

FIGURE 9. Differential Cell Count in Bronchoalveolar Lavagate up to 18 Months of Exposure to Toner and TiO_2 in Syrian Golden Hamsters. Aerosol Concentrations as Listed in Table 3.

influenced by the sex difference in body weight and a resulting difference in the minute volume.

Generally, tracer clearance results in hamsters showed a larger intra-group variability than observed in rats. Similar observations were made on variability of various parameters such as body weight, lung weight and retained mass in lungs. In spite of these statistical fluctuations it is clearly shown that TiO_2 and toner led to a retardation of the alveolar clearance.

The different behavior between hamsters and rats can also be seen in the differential cell count in the bronchoalveolar lavage (compare Figure 9 to Figure 5). Results show an increase of the number of lavagable alveolar macrophages and substantial increase in PMN.

In a recovery experiment after 9 months, female hamsters were removed from the inhalation chambers in the groups exposed to toner medium and high and TiO_2. Animals were kept in filtered air for up to a further 6 months. The retained mass was analyzed after 3 and 6 months. The retained mass and calculated half-time of alveolar clearance are presented in Table 6. For TiO_2 exposure,

TABLE 6

Retained Mass at 9 Months of Exposure and Calculated Half-time of Alveolar Clearance (with 95% Confidence Limit) of the Retained Material in a Post-Treatment Observation Period of 6 Months in Female Hamsters in Study E

Nominal aerosol concentration [mg/m³]	Retained mass [mg/lung]	Half-time of alveolar clearance [days]
		mean (95% Confidence limit)
6/16 TONER	0.27 ± 0.04	114 (63 - 617)
24/64 TONER	1.34 ± 0.51	359 (129 - ∞)
40/30 TiO_2	10.3 ± 1.67	441 (271 - 1181)

alveolar clearance was retarded, as shown by the half-time of 441 days. Using labelled tracer particles a clearance half-time of 641 days was calculated (Table 3). In the toner high exposure group, the clearance half-time was 359 days compared to 246 days obtained by the tracer method. Although in some cases sex differences in the retention and clearance behavior in hamsters were observed, these data show a considerable congruence.

Chronic Inhalation Study of Diesel Engine Exhaust, Carbon Black and TiO_2 (Anatase) in Wistar Rats and NMRI and C57BL Mice (Study D)

The exposure concentrations in this study for the carbon black and the TiO_2 exposure groups were changed twice, as indicated in the footnote to Table 2, Study D. The reason for these changes was the same as mentioned in the chronic inhalation study of toner in hamsters. Interim retention measurements had shown that the desired similar lung burden in the diesel high, carbon black and TiO_2 exposure groups would not have been reached by the original values (Heinrich et al. 1989).
The quantities of retained masses in lungs after 4.5 months of exposure and the corresponding half-times of the alveolar clearance are presented in Table 2. These values are transferred also to Figure 2, showing the same pattern of impairment of the alveolar clearance with dependence on the retained mass. The time sequence of the retention values is presented in Figure 10.
After 22 months of exposure, the lung weight increased by 1.17, 1.74, 4.37, 5.1 and 4.25 in the three diesel exposure groups, the carbon black and TiO_2 exposure groups, respectively. This substantial elevation of lung weight in the high dose groups led to changes in the mechanical behavior of the lung followed by altered breathing and particle deposition pattern during the course of the study. Mechanical lung function measurement of the rats documented a shallower breathing pattern and a less compliant lung.
Clearance measurements using ^{85}Sr-polystyrene particles in the further course of the study also indicated this change in the deposition pattern caused by the considerable increase in lung weight. Therefore, the correlation of the retained dust volume in lungs and the clearance rate of these highly exposed rats did not follow the same pattern as listed in Figure 2. As shown in Figure 11 a high particle load of the lungs accompanied by a considerable increase in the lung weight due to various exposure-related tissue reactions may lead to an altered deposition pattern of the inhaled particles. At this

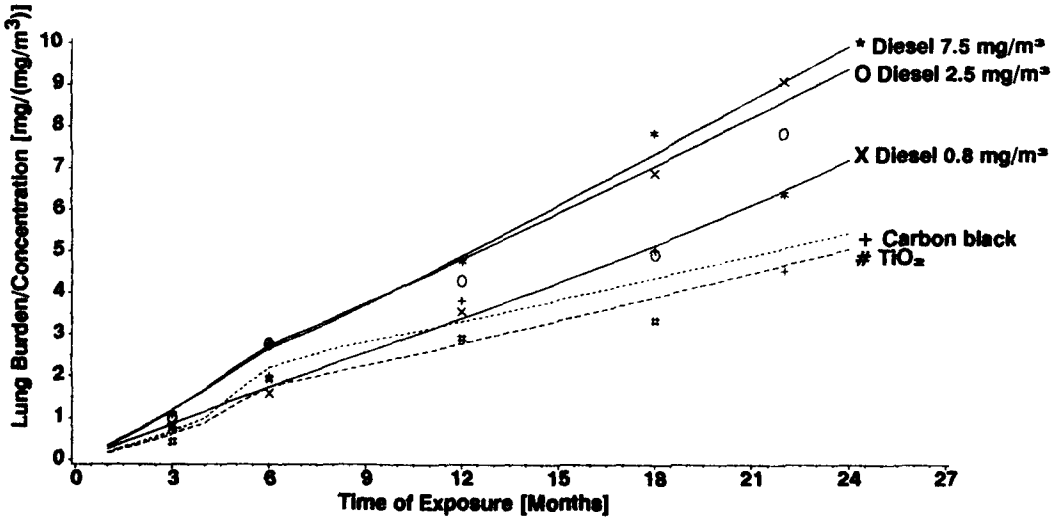

FIGURE 10. Lung Burden of Test Materials of Diesel Soot Particles, Carbon Black and TiO_2 in Female Rats Normalized to the Exposure Concentration (Study D). Lines: Model Calculation

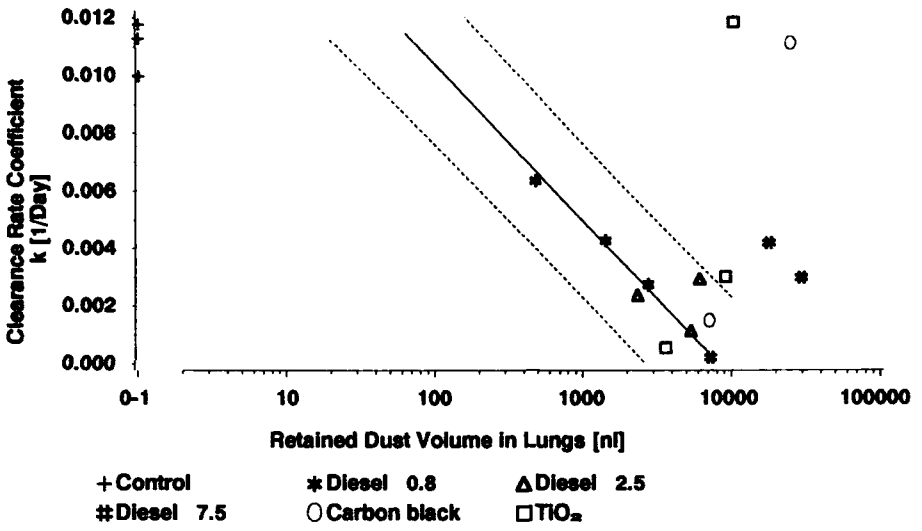

FIGURE 11. Clearance Rate Coefficient of Labelled Particles (^{85}Sr-Polystyrene) as a Function of the Retained Dust Volume Measured after 3, 12, and 18 Months of Exposure. Apparent Normalization of Alveolar Clearance at High Lung Burden (For Detail See Text). Chronic Inhalation of Diesel Exhaust, Carbon Black and TiO_2 in Rats.
Lines: Regression Curve and 95% Confidence Limit from Figure 3.

point, a much higher fraction of the inhaled material may be deposited in the bronchi resulting in a faster clearance.
This effect had also to be taken into account for the model calculation of particle retention in Figure 10 where a substantial decrease of the deposition rate had to be presumed for carbon black, TiO_2 and for the medium and high diesel exposure groups after 6 months of exposure.
An interstitial fibrosis was observed at serial sacrifices at 12 and 18 months in the carbon black, TiO_2 and diesel high exposure groups.
Retention values of mice principally followed the same pattern as those found in the rat. No steady-state was reached during the exposure period (see Figure 12). Assuming first order kinetics the equation $m = D/k \cdot c \cdot (1-e^{-kt})$ was fitted to the retention values. The deposition rate D and the clearance rate constant k was estimated by a non-linear regression using lung burden m, aerosol concentration c and exposure time t of individual animals. The resulting half-times of diesel retention which were above 500 days are shown in Figure 12. This means that the phenomenon of lung overloading can also be observed in mice.

Effects of particle size

Besides the amount of retained volume there are other factors which also influence the response of the lung. Although the mass median diameters of anatase and rutile were similar, the ratio of retained mass divided by the cumulative exposure dose was considerably higher for anatase. This faster uptake may be partly influenced by a disaggregation of the anatase dust into small primary particles in the lung (Takenaka et al. 1986) which also led to higher inflammatory reactions (Oberdörster et al. 1990).

Effect of animal age

Repeated measurements of the alveolar clearance using ^{85}Sr-labelled polystyrene particles in rats unexposed to dust (control) during their lifespan had consistently shown a slight but statistically significant decrease in alveolar clearance. Typical values at 5 months of age showed a

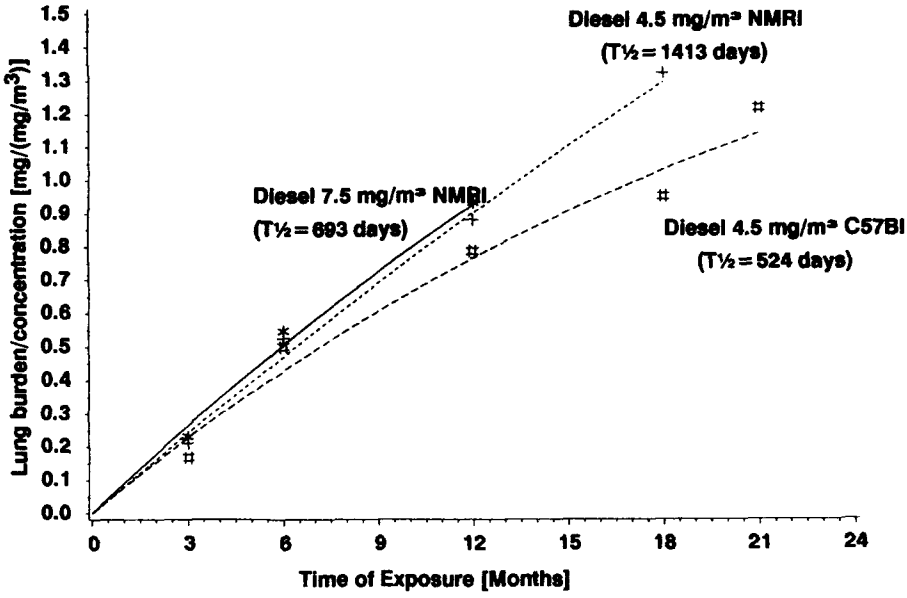

FIGURE 12. Lung Burden of Diesel Soot Particles in Female NMRI and C57BL Mice. Lines According to a First Order Kinetic Model Calculation with Corresponding Half-Times of the Lung Clearance of Diesel Soot Particles.

half-time of 45 days, whereas after 23 months the values increased to 74 days. This is documented by the control values in Figure 2, where the square represents the results from 11-month-old animals and the triangle the results of 23-month-old rats.

DISCUSSION

Accumulation of large quantities of insoluble material in the lungs, clearance impairment and inflammatory response are some of the characteristic signs of lung overloading, a phenomenon which has been discussed by Morrow (1988). In the F-344 rat, lung overloading is generally reached in the range of 0.5 - 1.5 mg or approximately 1 mg of material/g of lung tissue. Since phagocytosis of particles with low solubility and low biological activity depends primarily on their size and not on their density, the most appropriate manner to compare various dusts appears to be the volume of material (Morrow and Mermelstein, 1988).

Similar results of lung overloading were observed upon prolonged exposure of rats to diesel exhaust (Vostal et al. 1982, Chan et al. 1984, Wolff et al. 1986, Heinrich et al. 1986). Vostal (1986) suggested that there is a threshold between cumulative exposure (concentration x time) and particle clearance "overload". As indicated above, it appears to us that a similar threshold relationship should exist between particle clearance "overload" and the onset of fibrosis. The induction of fibrosis after high exposure to TiO_2 was also reported by Lee et al. (1985).

Our results using various test materials and three species indicate that dust overloading of lungs appears to be a generic phenomenon, observed upon over-exposure of the lungs to various particles of low solubility and low acute toxicity.

Characteristic findings of dust overloading of lungs are: a) alveolar clearance retardation of tracer particles, b) increased retention of test materials in the lung, c) increase in lung weight, d) accumulation of dust-laden macrophages, e, persistent inflammation, f) increased epithelial permeability, g) elevated infiltration of neutrophils, h) increased transfer of material to the lung-associated lymph nodes, e) onset of fibrosis after a

critical dose (time-integrated concentration) and sufficient time interval. The phenomenon of dust overloading must be taken into account in extrapolating from the highest exposure levels to ambient levels of exposure relevant to man. Effects observed at the high level may be uniquely related to the accumulation of a high burden of insoluble particles. Accordingly the demonstration of "lung overloading" in the various studies indicates that caution should be used in extrapolating the prevalence of lung fibrosis in rats exposed to a high level of dust, to potential fibrogenicity in humans exposed to much lower levels of the same dusts. On the other hand, as dust overloading seems to be a generic phenomenon and taking into account the longer clearance half-time of insoluble particles in humans compared to rodents, it cannot be excluded that a high dust exposure may lead to similar effects in humans as observed in rodents. It should be noted that the effect of dust overloading was also partly observed at exposure concentrations below 10 mg/m^3 respirable dust, i.e. within the range of the occupational dust standards. Assuming that there is a human counterpart to the dust overloading phenomenon at the same lung burden (milligram dust per gram lung), extrapolation modelling can be used to show that the current occupational dust limits do not protect workers sufficiently (Morrow et al. 1991).

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Developments in Modeling Alveolar Retention of Inhaled Insoluble Particles in Rats

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ABSTRACT

The development of a non-linear, physiology-oriented compartmental kinetics model of clearance and retention of insoluble particles in the alveolar region of rat lungs is described. The model recognizes the dominant role of the alveolar macrophages in alveolar clearance and retention and assumes that, eventually, increasing burdens of phagocytized particles impair the mobility of alveolar macrophages. Thus, the macrophage-mediated particle removal process will be retarded. In continuous inhalation exposures, this may cause the sequestration of heavily loaded macrophages and a general overloading of the alveolar region of the lung with retained particles. A basic model design accounting for macrophage life time, phagocytosis rate, mobility decline and limited load capacity was applied for the simulation of experimental data of several chronic and subchronic inhalation studies. The results were very good, but some of the model parameters did not comply with the self-imposed quality criteria. Apparently, the average load of the macrophage pool was an insufficient parameter to account consistently for sequestration. The model was then revised and features now particle load distributions in the macrophage pool. Preliminary efforts to simulate the same experimental inhalation data as before gave again very good results and the model parameters utilized did no longer show the previous inconsistencies.

INTRODUCTION

For many years in the past, experimental clearance and retention of insoluble particle deposits in the respiratory tract have been characterized by a simple mathematical model of first-order kinetics. The reduction dM of the mass burden M in a particular lung compartment during the time differential dt was assumed to be proportional to the compartmental mass burden:

$$-dM = kMdt \tag{1}$$

Then, for a deposition rate $D(t)$, a simple kinetic equation

$$\frac{dM}{dt} = D(t) - kM \tag{2}$$

is obtained.

The constant constant k provides a simple analytical solution featuring a diminishing exponential time-function which, for constant chronic exposure conditions, (i.e. $D(t) = D$), establishes a steady-state mass burden, $M_{max} = D/k$, and leads eventually to complete elimination of the mass deposit after an extended post-exposure period, where $D(t) = 0$. Generally, the transfer rate constant k is used as a convenient characteristic parameter relating the clearance of a lung compartment to an apparent biological half-life of the compartmental mass deposit

$$t_{1/2} = \frac{\ln 2}{k}. \tag{3}$$

However, this model is not based on any particular insight into the underlying physiological clearance mechanisms. The first-order kinetics model is a very elementary and empirical characterization of the clearance process. Thus, it is applicable to both tracheobronchial and alveolar lung clearance, although the actual physiological clearance mechanisms in these two regions are entirely different. In general, first-order kinetics models are justified as long as the mass transfer rates depend actually on an invariable physiological transport mechanism, like a steady velocity of the mucous layer in the bronchial tree or an inherent mobility of the alveolar macrophages on the alveolar surface. This condition appears to be satisfied when the mass burdens of the respective compartments remain small. However, for increased mass burdens as they may occur at high exposure concentrations, non-linear effects seem to play a key role in explaining the observed effects. These effects show that, eventually, the mass transfer rate constants depend on actual compartmental mass burdens.

Nevertheless, first-order compartmental models of empirical retention data have been surprisingly successful for moderate exposures, and compartmental clearance and retention models using constant transfer rate coefficients still prevail in the literature.

There is no doubt that half-lives on the order of hours relate to tracheobronchial clearance. Bronchial deposits are cleared by an effective and specific mechanism in which the relatively fast motion of the bronchial mucous layer is mediated by the ciliated epithelium of the conductive airways. In contrast, the pulmonary region has no such mechanism, and clearance half-lifes exceeding the order of a day are historically assigned to particle elimination from the pulmonary region.

In the pulmonary or alveolar region, removal of insoluble particles is strongly dependent on endocytosis and a relatively slow cellular transport. Alveolar macrophages, although primarily equipped for defenses against biological foreign matter, play a key role in the alveolar clearance of particles.

The classical compartment model of the Task Group on Lung Dynamics (1966) acknowledged the carrier function of the alveolar macrophages and assigned two transfer routes of different rate to the macrophage-mediated clearance, but with this option, the model relied entirely on competing first-order kinetics, most likely because, in the past, this assumption had been so successful empirically. Even in 1981, when Soderholm first suggested to use a sequestration compartment in order to account for the apparently irreversible retention of significant fractions of heavy alveolar particle deposits, the kinetics were still of first order. Actually, non-linear kinetics were not applied in a lung retention model until four years later, when Smith (1985) published a model borrowing from Michaelis-Menten kinetics for the macrophage-mediated alveolar clearance processes.

Today's growing data base in biochemistry and pulmonary physiology of the lung presents an increasing challenge for an attempt to postulate physiological mechanisms of alveolar clearance which would be able to explain the empirical data for particle clearance and retention by applying results obtained in the fields of pulmonary cell physiology and biochemistry.

This paper reports on a succession of new non-linear modeling efforts (Stöber et al., 1989; Stöber et al., 1990; Stöber and Koch, 1990). The basis of our physiology-oriented compartmental kinetics model relates to an intuitive postulate published by Morrow (1988) who concluded from inhalation studies under overload conditions that, in such a case, the macrophages may

reach their finite particle load capacity, and with increasing particle load, their mobility will steadily decline until they are immobilized. Furthermore, we account for the simultaneous effects of phagocytic particle collection on one hand and particle release by macrophage death on the other, which seems to have a significant impact on the retention.

THE PHYSIOLOGY-ORIENTED COMPARTMENTAL MODEL OF ALVEOLAR RETENTION OF INSOLUBLE PARTICLES

At the outset, we would like to emphasize that the versions of the model described here refer to insoluble particles only. This is primarily for the sake of simplicity. It would not be too difficult to extend the model to account simultaneously for the dissolution rate of slowly dissolving particles in different environments of body fluids. However, this would merely add to the number of parameters and their uncertainties. Thus, at this stage, an inclusion of solubility did not appear to be desirable.

However, there is a more stringent limitation which cautioned us to confine our model applications to (Fischer 344) rats only. Experimental results indicate that the behavior and the magnitude of the model parameters may depend on the animal species. For instance, the clearance mechanism in the hamster lung seems to be significantly different from the rat (Bailey et al., 1985). This, by the way, sheds some light on the delicate question as to whether or not retention models for animals may be applicable to man.

In addition to the Morrow hypotheses, there was another important aspect: estimates of the life time of alveolar macrophages in rats are reported to range from less than one week to, at most, five weeks, while the alveolar clearance by macrophage-mediated transfer to the tracheobronchial tract has a half-time of, at least, six weeks. This surely indicates that the clearance process must involve more than one macrophage generation. In other words, at least a fraction of the macrophages do not leave the alveolar surface during their life time and will perish on the alveolar surface. Actually, phagocytosis of the particle load of dead phagocytes by viable macrophages was already described many years ago by Heppleston (1963). Although this must have a significant effect on the transfer kinetics, so far no model has accounted for this phenomenon.

We expanded these observations into a model of the life-pathway of alveolar macrophages in rats. Experimental evidence indicates that, at most times, the alveolar macrophage population is in a quasi-steady state. Irritation and inflammation of the alveolar region appear to activate additional alveolar macrophages and transient phagocytes, such as neutrophils and other polymorphonuclear leukocytes, but increases in population size are not unlimited. In chronic inhalation studies, the dominating macrophage population will probably quickly attain a new steady state at an elevated level.

Since alveolar macrophages of the pool are continuously lost by death or by transfer to other locations, the steady-state population is in dynamic equilibrium, and losses will be compensated by recruitment of new alveolar macrophages. Figure 1 gives a general scheme of the macrophage life-pathway as it is used for the model.

We assume that a pool of N viable alveolar macrophages will have a continuous influx of new macrophages at a recruitment rate $\dot{N}$. At steady state, this influx will compensate for the macrophage death rate, ρN, and for losses by transfer to the tracheobronchial tract, $\kappa_p N$ (which constitutes the primary alveolar clearance route), and, tentatively, transfer to the lymphatic system, $\lambda_p N$, and to the interstitial space, $\epsilon_p N$.

If the transfer rate coefficients total

$$\phi_p = \kappa_p + \lambda_p + \epsilon_p \tag{4}$$

then the steady-state of the macrophage population requires

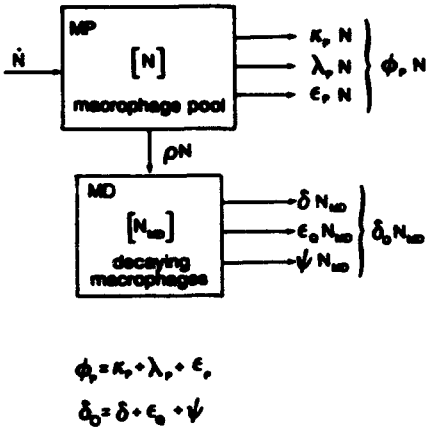

FIGURE 1.
Life-pathway of macrophages in the compartmental model for insoluble particles deposited in the lung.

$$\dot{N} - (\rho + \phi_p)N = 0 \tag{5}$$

and with the normally invariable median life time of the macrophages,

$$\tau_L = \frac{1}{\rho} \tag{6}$$

we obtain

$$N = \frac{\tau_L}{1 + \phi_p \tau_L}\dot{N} = \tau_R \dot{N} \tag{7}$$

where

$$\tau_R = \frac{\tau_L}{1 + \phi_p \tau_L} = \frac{1}{\rho + \phi_p} \tag{8}$$

is the median residence time of viable macrophages on the alveolar surface.

We assume that this steady-state mechanism will continue regardless of the particulate lung burden. For significant lung burdens, the recruitment rate, $\dot{N}$, will increase quickly to $\dot{N}_{max}$ and bring the total population N to a saturation level, N_{max} (Adamson and Bowden, 1981).

In the meantime, we checked two versions of the new retention model for their simulation capability, and for all suitable experimental studies selected for the tests, we obtained very reasonable simulations by consistently using a death rate constant of $\rho = 0.14$ d^{-1} corresponding to a macrophage life time $\tau_L = 7$ days. Another consistent value was the clearance rate $\kappa_p = \phi_p = 0.015$ d^{-1} ($\epsilon_p = \lambda_p = 0$). For these values, Equation (8) shows that the residence time of the macrophages on the alveolar surface would vary only by as little as 10 %, even if the mobility of the macrophages would decline completely ($\phi_p = 0$). This finding is in keeping with experimental evidence of a rather constant population of alveolar macrophages in the rats. In the activated state of irritation or inflammation by the continued presence of foreign particles, values of $N = 25 * 10^6$ were reported in the literature. As $N = \dot{N}\tau_R$, the macrophage recruitment rate, at this stage, will be at a maximum value of $\dot{N} = 3.57 * 10^6$ d^{-1}.

The macrophage life-pathway model further implies that the dead macrophages are not immediately removed from the alveolar surface. Instead, they constitute a separate compartment of non-viable macrophages on the alveolar surface. This compartment, MD, is assumed to be proportional in size, N_{MD}, to the macrophage population, N. A ratio of $N_{MD}/N = \rho/\delta_0 = 0.05$ was used, which relates to the fact that about 95 % of lavaged macrophages are found to be viable. Then, the inverse of the decay rate constant, δ_0, which is the invariable decay time, τ_D, amounts to 8.4 hours.

The crucial assumption when applying the macrophage life-pathway scheme to our retention model is the mode of redistribution of particles contained in the decaying macrophages: the retention model assumes that the decaying macrophages clear the alveolar surface by lysis (δ), as well as by resorption into the interstitial space (ϵ_Q) and by sequestration (ψ). This occurs in such a way that the respective rates are determined by the particle burden, but they also must meet the requirement that the sum

$$\delta_0 = \frac{1}{\tau_D} = \delta + \epsilon_Q + \psi \tag{9}$$

remains invariable.

The complete retention model with competitive transfer routes is shown in Figure 2. The alveolar subcompartments are shown inside the dashed-line rectangle, and the separate recipient compartments, i.e. the tracheobronchial tract and the lymphatic system, the latter representing, in essence, the lung-associated lymph nodes, are located outside. The mass deposition rate, $\dot{M}_s$, brings the particles first to the alveolar surface from where they will be redistributed. The appropriate transfer rate coefficients are shown along the transfer lines to the other compartments. These coefficients are to be multiplied by the particle mass of the releasing compartment to give the mass transfer rate. The coefficients κ_s, ϵ_s, and λ_s imply a transfer of free particles. However, the coefficients α (phagocytosis), κ_p (classical alveolar clearance), ϵ_p (transfer into interstitium) and λ_p (transfer to the lymphatic system) are macrophage-mediated translocations which are assumed to be dependent on macrophage mobility. According to Morrow (1988), this mobility declines with increasing macrophage load.

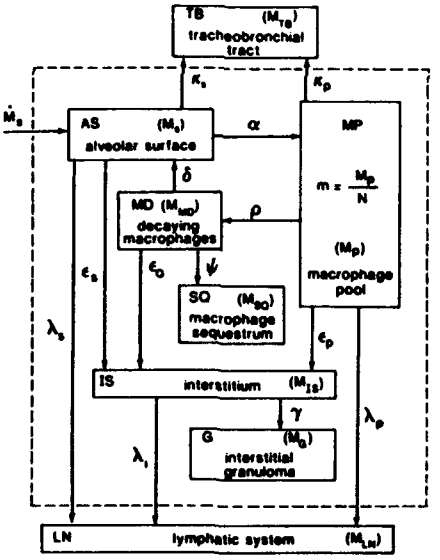

FIGURE 2.

Diagram of the compartments and transfer rate coefficients used in the original alveolar retention model

To accomplish this decline by the model, we employed a variety of arbitrary mobility coefficients, f or f_m, all of which were monotonic functions of the particle mass load of the macrophages. The functions declined from unity at no load or at a critical lower load, m_{cf}, and ended with zero at the increased load at which mobility vanished. Initially, this was at the maximum load capacity, m_{max}, of the macrophages. All of the simulations of the actual data were finally made with a function having a general pattern as graphically shown in Figure 3.

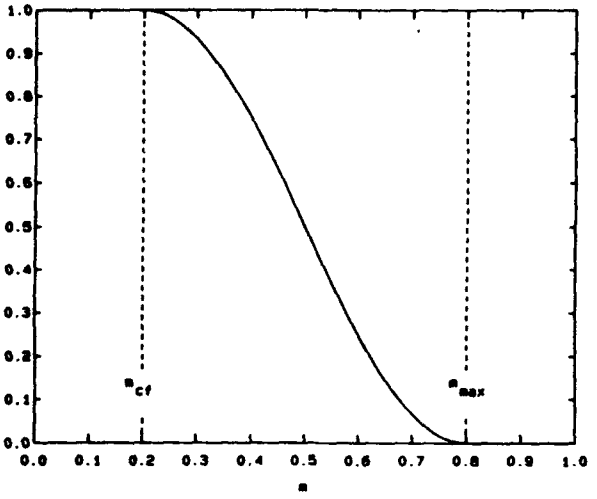

FIGURE 3.

General pattern of the adjustment function, f_m, describing the decline of macrophage mobility as dependent on the mass load of particles in the macrophage. The characteristic values, m_{cf} and m_{max}, are to be determined by best fit to experimental retention data.

Mathematical details are given elsewhere (Stöber et al., 1990). In the model the actual values of m_{cf} and m_{max} turned out to be related to the inhaled material and were determined by best fit to all of the data of the selected sets of inhalation studies using the same material.

The scarcity of quantitative data on the distribution of the particle mass in the various internal alveolar compartments made it mandatory to strip the conceived original model and its competitive transfer routes to the essential features. This simplified model is represented in Figure 4. Compared to Figure 2, the more tentative transfer routes characterized by the transfer rate coefficients $\kappa_s, \lambda_s, \lambda_p, \epsilon_p$ and ϵ_Q were eliminated prior to the simulation tests. Furthermore, the actual tests indicated that all final simulations were compatible with the assumption of $\gamma = 0$ (Stöber et al., 1990). This rendered the interstitial granuloma compartment dispensable and left the macrophage sequestrum as the only compartment accumulating irreversible deposits.

In addition, the invariance of Equation (9) will be reduced by the simplification to

$$\delta_0 = \delta + \psi. \tag{10}$$

Then, by defining a function, h or h_m, describing the change of the sequestration rate coefficient according to the macrophage load, m, by

$$\psi = \psi_{max} h_m \tag{11}$$

the appropriate change of the lysis rate coefficient, δ, would automatically follow as

$$\delta = \delta_0 - \psi_{max} h_m \tag{12}$$

The function, h_m, must increase monotonically from zero at no macrophage load or up to a critical load, m_{ch}, reaching unity at a specific load where the sequestration rate coefficient is at its maximum, ψ_{max}. Obviously, this value cannot exceed the value of the decay rate coefficient, δ_0. The specific load where ψ_{max} is reached was chosen to be the maximum load capacity, m_{max}, of the macrophages. Figure 5 shows the general pattern of the selected arbitrary function used for the actual data simulations (Stöber et al., 1990). Again, the parameters m_{ch} and m_{max}

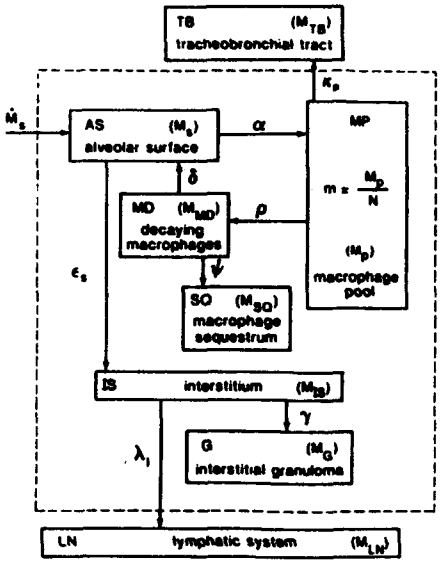

FIGURE 4.

Simplified version of the original alveolar retention model shown in Figure 2. The transfer routes are reduced to the essentials. Except for the alveolar surface compartment where the life-pathway transfers of the macrophages return particles by macrophage lysis, the compartments have generally only one feeder route.

were related to the inhaled material and should be invariable for any suitable study using this material. In addition, of course, the value of m_{max} had to be the same as in function f_m. Full details of this simple version of the model are described in the paper repeatedly quoted above.

SELECTION OF STUDIES FOR TESTING THE SIMULATION QUALITY OF THE MODEL

To test the simulation capability of the new model, suitable experimental inhalation studies had to be selected, which would provide empirical data for, at least, the alveolar lung burden of insoluble particles during and after chronic or subchronic inhalation exposure. Additional information was desirable for the build-up of particle deposits in the lung-associated lymph nodes.

On the basis of these minimum criteria, five inhalation exposure studies conducted with Fischer 344 rats in three different laboratories were selected. The results of these studies pertain to three different insoluble materials, and all of the reported retention data were analyzed with the new model.

After properly setting the exposure parameters of the model to the respective values required, the model was expected to represent the empirical data of all of the studies by one typical set of parameter values. Variations of this set would be acceptable only if they reflected changes of relevant properties of the actually inhaled particulate matter.

Diesel Exhaust Inhalation

Two of the selected studies investigated the effects of inhaled diesel exhaust. Strom et al. (1988) described subchronic exposure experiments conducted at the General Motors Research

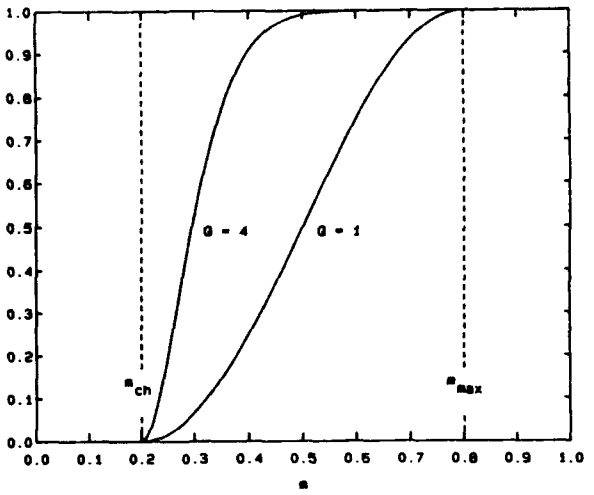

FIGURE 5.

General pattern of the adjustment function h_m, describing the rise of macrophage sequestration as dependent on the mass load of particles in the macrophage.

Laboratories (GMRL), while Wolff et al. (1987) of the Inhalation Toxicology Research Institute (ITRI) in Albuquerque reported on a chronic exposure study. In these studies, the carbonaceous cores of the diesel soot represented the insoluble aerosol material retained in the rat lungs. During deposition, however, the soot carried a certain amount of extractable organics into the lungs.

The subchronic GMRL study used 4 different initial exposure periods of up to 84 days at concentrations of 6 mg/m^3 exhaust particles and measured post-exposure clearance rates during the subsequent year. Retention data for alveolar burden and lung-associated lymph nodes were obtained. The ITRI study gave alveolar lung burden data for 3 exposure concentrations of up to 7 mg/m^{3}exhaust particles for two years of exposure.

Carbon Black Inhalation

One of the other selections for simulation was a recent GMRL study by Strom et al. (1989), who used commercially available carbon black in subchronic inhalation tests with 3 different initial exposure periods of up to 41 days at 6 to 7 mg/m^3 carbon black particles followed by one-year recovery periods. Data for the alveolar lung burden and for the lung-associated lymph node load were obtained.

Test Toner Inhalation

The two remaining selections were studies conducted at the Fraunhofer-Institute of Toxicology and Aerosol Research (Fh-ITA) in Hannover by Muhle et al. (1988; 1989). In these studies, rats were exposed to aerosols of a specifically prepared test toner powder.

In the first study, the rats were exposed for 24 months to three different test toner concentrations extending up to 16 mg/m^3. The second study comprised subchronic 90-day exposures to two different toner concentrations, with subsequent clearance measurements over a recovery period of 18 months. Both studies gave data on the particle burdens of the lung and the lung-associated lymph nodes.

ANIMAL AGE RELATED ADJUSTMENTS OF THE MODEL

Considering the biological nature of the transfer processes, many of the associated rate constants, conventionally assumed to be constant or depending merely on the load of the macrophages, could genuinely depend on animal age. This could possibly restrict the applicability of the model unless known effects of this kind are accounted for.

The authors of the selected studies for the simulation tests discussed actually three effects which explicitely or by implication did or could depend on animal age.

A straight-forward case is the recent finding of an inherent decline of the alveolar clearance rate with animal age in Fischer 344 rats. This was reported and reconfirmed by Muhle and coworkers (1989). The data in the control groups of chronic life span studies with test toner indicated a decline of the clearance rate over the life time by some 40 %. This would certainly have an impact on the pattern of the macrophage mobility decline under particle load. Therefore, the model included a time dependent adjustment, f_t, which was a practically linear relationship, as a term of the mobility coefficient which was then

$$f = f_t f_m. \tag{13}$$

Figure 6 shows the new time dependency and some experimental values obtained by Muhle and coworkers.

Furthermore, all authors considered the ventilation rate of the animals in their studies. This would make the deposition rate, $\dot{M}_s$, dependent on age, because younger animals have a lower minute volume of ventilation than older ones. However, the divergence of the reported data suggested that, prior to the simulation tests, the model should not be tied to a selected data set. Instead, the deposition rates were taken as constant, time averaged values which are directly proportional to the specific exposure concentration in each particular study.

Only two of the studies were chronic investigations over the life span of the animals. There was a total of six different exposures. One half of these were exposures to diesel exhaust reported

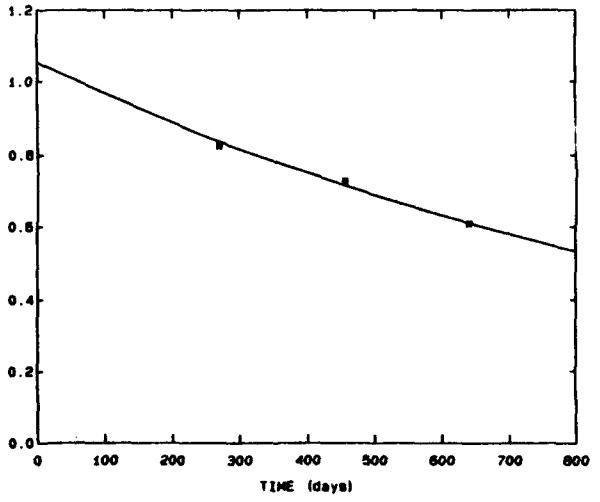

FIGURE 6.

Pattern of adjustment function, f_t, established by exponential fitting to experimental data obtained in control rats with Sr^{85}-labeled test aerosols by Muhle et al., 1988. The function describes the decline of macrophage mobility as dependent on time (i.e., animal age).

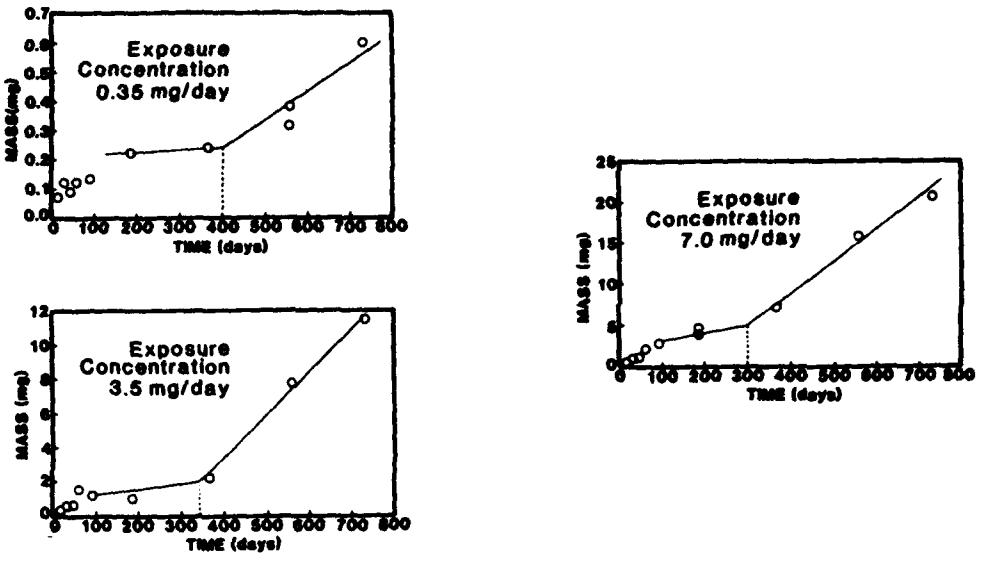

FIGURE 7.

Interpretation of the experimental data of the chronic diesel exhaust inhalation exposures at ITRI (Wolff et al., 1987) for different airborne diesel soot concentrations. The impairment of clearance seems to show after 300 to 500 days of exposure.

by Wolff and coworkers. Thus, their surprising finding of a time dependent onset of a severe impairment of clearance after about one year of exposure weighed heavily and its impact on our model was carefully evaluated. Figure 7 shows the experimental data. They indicate that the clearance loss may have happened even when the total alveolar load was below 0.3 mg per animal. A crude examination of the experimental data in the three exposures indicates that, after one year of exposure, the lung burdens began to show an almost linear increase that continued during the remaining exposure period. The influence of the total alveolar load on the latency period appears to be relatively weak, considering that a 20-fold increase in exposure concentration shifted the onset of loss of alveolar clearance rate from somewhere below 500, to some 300 days, after the beginning of exposures.

These apparently time dependent clearance breakdowns finally persuaded us to introduce a time dependent function, h_t, to bring the adjustment function for the sequestration rate coefficient to

$$h = h_t h_m. \tag{14}$$

The new adjustment term, h_t, is then an arbitrary monotonic function in time with characteristic time parameters, t_c and t_{SQ}. The general pattern is the same as shown in Figure 5 after replacing the mass scale of the abscissa and the mass parameters m_{ch} and m_{max} by a time scale with the time parameters t_c and t_{SQ}, respectively. With this somewhat empirical remedy, the model should be able to cope with the chronic diesel exhaust exposure data, but, in contrast to the other adjustments, this one is philosophically poorly justified as it apparently does not relate to a time-weighted burden, $m * t$.

SIMULATION RESULTS WITH THE ADJUSTED SIMPLE MODEL

The potentially variable parameters of the simple model includes the time parameters for the phenomenological adjustment of the sequestration onset (Tabel 1).

The first data simulated by trial and error were from the four subchronic diesel exhaust

TABLE 1.

Model Parameters for the Simulation of Experimental Data

Life Time of Alveolar Macrophages	$\tau_L \rightarrow 1/\tau_L = \rho$
Turnover Time of Phagocytosis	$\tau_0 \rightarrow 1/\tau_0 = \alpha_{max}$
Ratio of Decay Time to Life Time of Macrophages	$F_r = \tau_D/\tau_L \rightarrow 1/\tau_D = \delta_0$
Macrophage-mediated Clearance to Bronchial Tree	κ_{pmax}
Transfer from Alveolar Surface to Interstitium	ϵ_s
Maximum Fraction of Decay being Sequestered	$S_r \rightarrow S_r\delta_0 = \psi_{max}$
Interstitial Transfer to Lymph Nodes	λ_i
Interstitial Transition to Irreversibility	γ
Maximum Mass Burden of Macrophage Pool	M_{pmax} $m_{max} = M_{pmax}/N$
Critical Macrophage Pool Load for Mobility Decline	M_{cf} $m_{cf} = M_{cf}/N$
Critical Macrophage Pool Load for Sequestration Onset	M_{ch} $m_{ch} = M_{ch}/N$
Exponent of Sequestration Increase by Load	Q $(Q \gg 1$: fast increase)
Critical Exposure Time of Sequestration Onset	t_c
Maximum Exposure Time of Partly Suppressed Sequestration	t_{SQ}
Exposure Conditions:	
Particle Mass Deposition Rate	$\dot{M}_s$
Duration of Inhalation Exposure	t_{exp}
Duration of Exposure Study	t_{end}

inhalation exposures reported by Strom and coworkers. Figures 8 and 9 display the final simulations of the eight data sets for either total alveolar burdens or lymph node loads.

The model parameters employed for these final simulations served then as the initial data base for simulations of the results of the other studies. The corresponding graph in Figure 10 shows the simulations for the chronic diesel exposure study. The same form of display is used

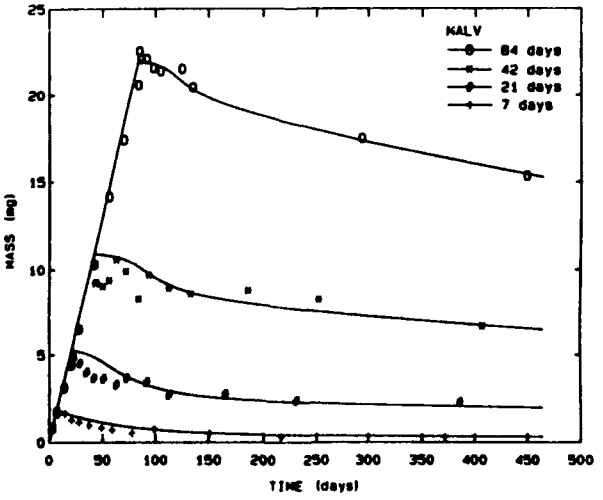

FIGURE 8.

Experimental data of total alveolar retention of diesel soot during and after subchronic diesel exhaust exposures (7 to 84 days) conducted by Strom et al., 1988, and corresponding data simulation curves of the simplified retention model.

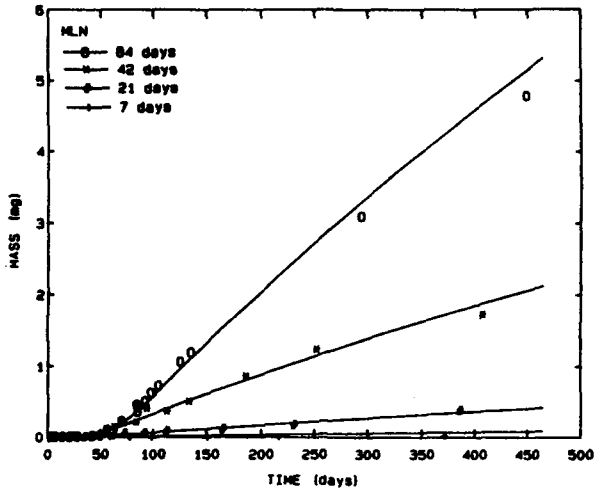

FIGURE 9.

Experimental data of lymph node retention of diesel soot during and after subchronic diesel exhaust exposures (7 to 84 days) conducted by Strom et al., 1988, and corresponding data simulation curves of the simplified retention model.

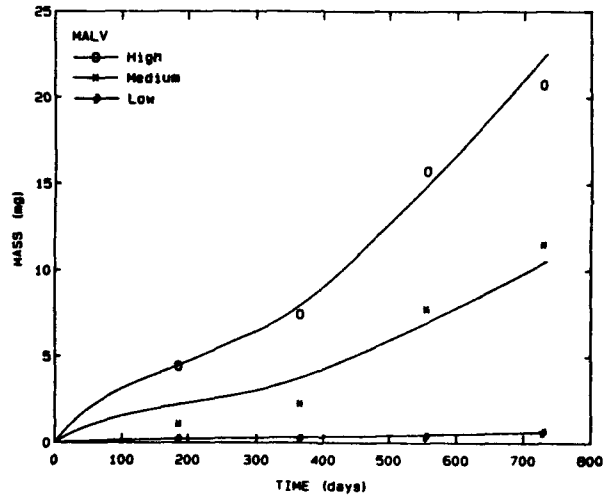

FIGURE 10.

Experimental data of total alveolar retention of diesel soot in chronic diesel exhaust inhalation exposures at ITRI (Wolff et al., 1987), and corresponding data simulation curves of the simplified retention model.

in Figures 11 and 12 for the simulations of the GMRL carbon black study. The simulations for the test toner data of the two Fh-ITA studies are shown in Figures 13 to 16. Tables 2 to 4 give the values of the model parameters that were used for the final simulations. The parameters in Table 2 were the same for all of the simulations. Parameters that were dependent on the inhaled material are listed in Table 3. Table 4 shows the specific exposure parameters for the five studies. At the bottom of Table 4, the characteristic time data for the sequestration adjustment function, h_t, are displayed. The latter parameters were the only ones that were influenced, not only by the inhaled material, but also as to whether the exposure was subchronic or extended over the life span of the rats.

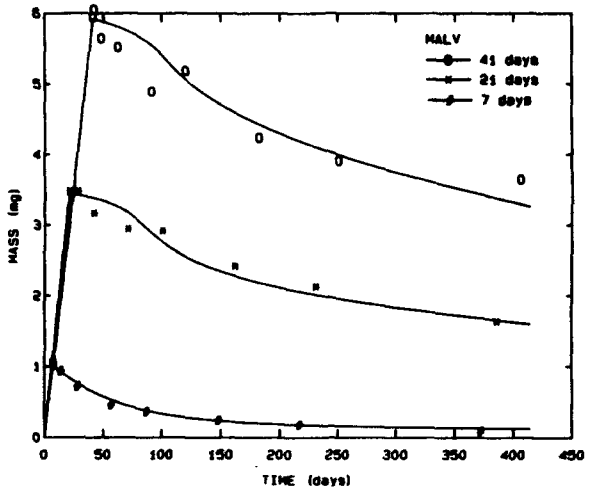

FIGURE 11.

Experimental data of total alveolar retention of carbon black during and after subchronic exposures (7 to 41 days) conducted by Strom et al., 1989, and corresponding data simulation curves of the simplified retention model.

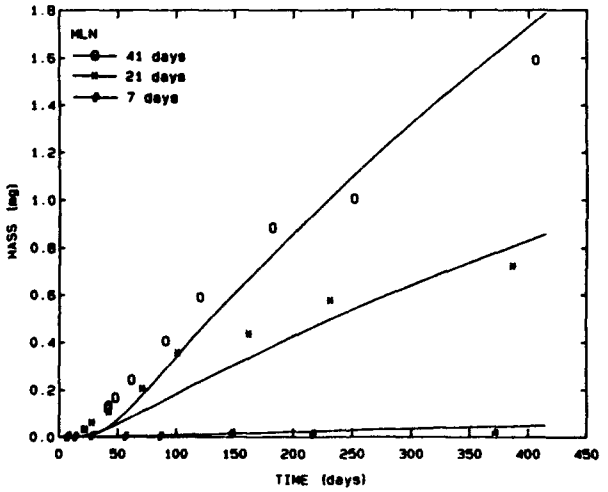

FIGURE 12.

Experimental data of lymph node retention of carbon black during and after subchronic exposures (7 to 41 days) conducted by Strom et al., 1989, and corresponding data simulation curves of the simplified retention model.

DISCUSSION OF THE FINAL SIMULATIONS WITH THE SIMPLE MODEL

As the graphical demonstrations in Figures 8 to 16 show, the new model was exceptionally successful in representing experimental retention and clearance data obtained under a great variety of inhalation conditions, ranging from modest to very severe exposures, with regard to both time period and aerosol concentration. As Table 4 indicates, exposure periods varied between one week and two years (subchronic GMRL studies versus chronic ITRI and Fh-ITA studies); exposure concentrations were changed by a factor of 40 (Fh-ITA studies), and deposition rates

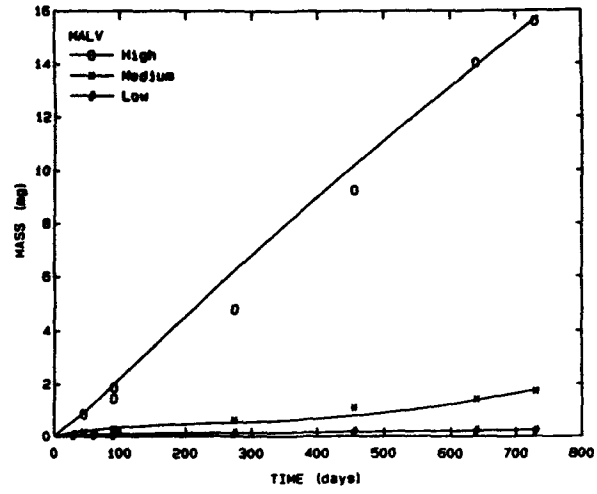

FIGURE 13.

Experimental data of total alveolar retention of test toner during and after subchronic exposures of 90 days as conducted by Muhle et al., 1989, and corresponding data simulation curves of the simplified retention model.

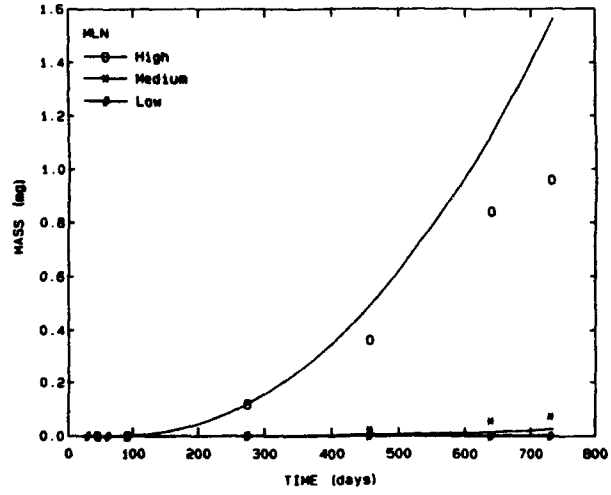

FIGURE 14.

Experimental data of lymph node retention of test toner during and after subchronic exposures of 90 days as conducted by Muhle et al., 1989, and corresponding data simulation curves of the simplified retention model.

changed by a factor of almost 200 (chronic test toner study versus subchronic diesel study). To cope with these changes, the model required few parameter changes that could not be explained by experimental design and exposure protocol.

According to Table 2, the trial and error approach permitted in all cases to leave the life time, τ_L, and the phagocytosis turnover time, τ_0, at constant values. These values are in keeping with literature data (Bowden, 1983). The same invariance could be maintained for the fraction, F_τ, of non-viable macrophages at a plausible value of 5 % (Oberdörster et al., 1990). Furthermore, as mentioned before, with $\gamma = 0$ in all cases, the interstitial granuloma compartment was actually

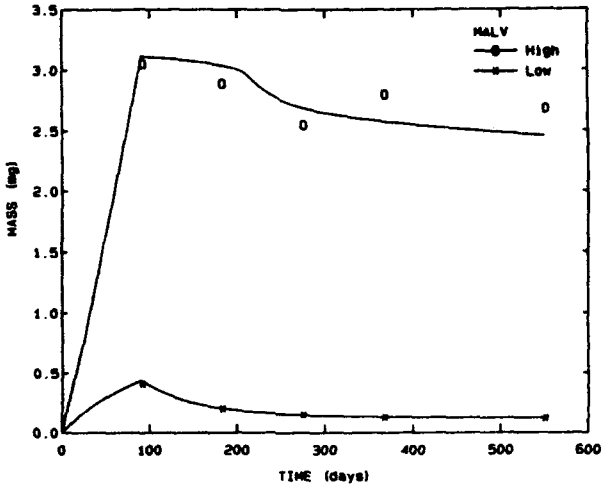

FIGURE 15.
Experimental data of total alveolar retention of test toner in chronic inhalation exposures at Fh-ITA (Muhle et al., 1988), and corresponding data simulation curves of the simplified retention model.

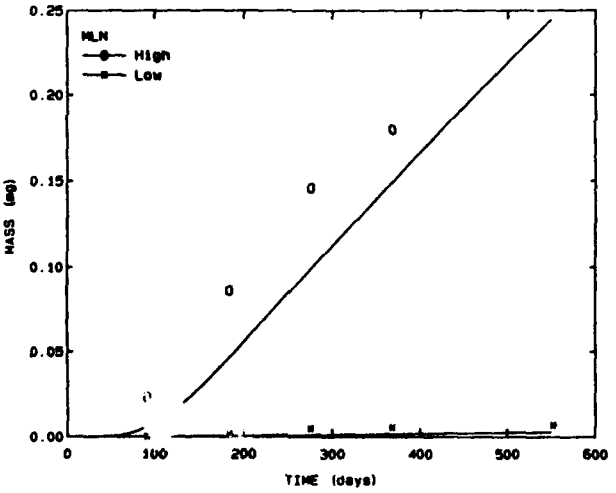

FIGURE 16.
Experimental data of lymph node retention of test toner in chronic inhalation exposures at Fh-ITA (Muhle et al., 1988), and corresponding data simulation curves of the simplified retention model.

TABLE 2.
Constant Model Parameters for all Final Simulations(Simple Model)

τ_L	τ_0	N	F_r	S_r	γ	M_{cf}/M_{pmax}	M_{ch}	Q
7 days	0.25 days	$2.5 * 10^7$	0.05	1.0	0	0.667	0	4

TABLE 3.
Model Parameters Varying with Inhaled Material (Simple Model)

		Diesel Exhaust	Carbon Black	Test Toner
κ_{pmax}	(day^{-1})	0.0132	0.015	0.014
ϵ_s	(day^{-1})	0.06	0.06	0.01
λ_i	(day^{-1})	0.001	0.0013	0.0004
M_{pmax}	(mg/lung)	4.5	1.3	0.8
M_{cf}	(mg/lung)	3.0	0.87	0.53

TABLE 4.
Model Parameters Varying with Exposure Conditions (Simple Model)

		Diesel Exhaust Inhalation Strom et al., 1988 Subchronic	Diesel Exhaust Inhalation Wolff et al., 1987 Chronic	Carbon Black Inhalation Strom et al., 1989 Subchronic	Test Toner Inhalation Muhle et al., 1989 Chronic	Test Toner Inhalation Muhle et al., 1989 Subchronic
Exposure Concentration	(mg/m^3)	6	0.35; 3.5; 7.0	6-7	1; 4; 16	10; 40
Exposure Conditions	$\dot{M}_s$ (mg/day)	0.275	0.0026; 0.026; 0.052	0.152; 0.168; 0.148	0.0015; 0.006; 0.024	0.0018; 0.036
	t_{esp} (days)	7; 21; 42; 84	730	7; 21; 41	730	91
	t_{end} (days)	375 - 460	730	375- 415	730	554
Characteristic times of Sequestration	t_c (days)	10	170	10	130	0
	t_{SQ} (days)	500	1000	900	5000	1000

dispensable. The constant ratio of m_{cf}/m_{max} (= M_{cf}/M_{pmax}, see Table 1) was higher than the value of 0.1 expected by Morrow (1988), but then, as shown below, the value of m_{max} behaved quite unexpectedly. The remaining parameters in Table 2 are associated with the sequestration pattern and their constancy is probably not significant. In the way we modeled sequestration, the degrees of freedom may have permitted this.

Table 3 shows the parameters which varied with the material inhaled in the respective studies. The variations among the alveolar clearance coefficients, κ_{pmax} (rather small deviations from a mean), as well as among the interstitial and the lymph node transfers, respectively, ϵ_s and λ_i (smaller values for bigger particles), appear quite plausible and were expected. However, it came as a big surprise to find the maximum load capacity of the macrophages, m_{max}, in this category. One of the foundations of the new model was the assumption that the load capacity of the macrophages would be proportional to the macrophage size (Morrow, 1988), and, thus, the average mass capacity would vary only to the extent that the bulk densities of the respectively inhaled particles were different. This, however, would not explain the difference found between the values for diesel soot and carbon black.

Finally, Table 4 reveals another, less surprising inconsistency of the model: the characteristic times, t_c and t_{SQ}, for the time pattern of sequestration show variations with changing exposure conditions. Considering the way this adjustment was introduced, the outcome is not surprising, but it is contrary to the self-imposed quality criterion.

These philosophical flaws notwithstanding an analysis of the values of the deposition rates (Table 4) and their actual alteration under the influence of different minute volumes of ventilation for young and adult rats indicated (Stöber et al.,1990) that the best fits of the model to the retention data would imply deposition data which were compatible and in good agreement with the experimental exposure and deposition efficiency data provided by the authors of the respective studies simulated.

Apparently, there were only two areas where the model was short of the required parametric performance. The more or less expected one was the dependency of sequestration on time which must be due to the empirical way we simulated this phenomenon. The other one concerned the

variations of the maximum load capacity which seemed to shake the foundation of the model. In hindsight, it was probably incorrect to assume tacitly that the total loss of mobility would not occur before the maximum load capacity was established. It is conceivable that the loss of mobility may well depend on the material the macrophages incorporate, and it may occur way before the load approaches the maximum volume capacity. This capacity would then be a separate entity and could still be the invariable macrophage parameter we envisioned. Thus, the parameter, m_{max}, of the model discussed so far may have represented the limit of mobility rather than the maximum load capacity of the macrophages.

In summary, the simplified model permitted simulations of high quality for all of the experimental studies tested. To the extent that some of the model parameters fell short of the implied consistency, the analysis opened two promising routes to further model improvements: by dissociating loss of mobility and maximum load capacity of the macrophages, the theoretical foundation of the model could be preserved, and an attempt to devise a better way of accounting for sequestration may remove the remaining inconsistencies.

THE DESIGN OF A REVISED RETENTION MODEL ACCOUNTING FOR MACROPHAGE LOAD DISTRIBUTION

The key problem of revising our retention model was the unsatisfactory representation of sequestered macrophages. It relates to a principal flaw of the model design which does not account for an actual distribution of particle loads among the macrophages. Histologically, sequestration is characterized by clusters of heavily loaded and apparently immobilized macrophages appearing on the alveolar surface (Strom and Garg, 1985). Thus, sequestration involves primarily macrophages with a load close to their load capacity limit. Therefore, an adequate model approach for assessing sequestration would have to account for the actual particle load distribution in the macrophages. Our simple model, however, was restricted to using the compartmental average load as the only parameter characterizing the macrophage pool. Thus, we made efforts to derive a macrophage load distribution in terms of the model parameters. A first approach of this kind led to an analytical integral expression under the assumption of a steady state of retention (Stöber and Koch, 1990), and the distribution pattern showed characteristics resembling a simple one-parameter approach by Yu and coworkers (1989).

However, considering the discontinuous nature of the phagocytosis process and the discrete number of particles representing the mass load in a macrophage, it should be possible to define a number distribution of uniform particles with a finite number of classes so that all classes could be treated as subcompartments of the macrophage pool. Similarly, the alveolar surface compartment would have subcompartments of particle clusters of various size on the alveolar surface as a result of the releases from the decaying macrophages. All of these subcompartments could then be included into the system of differential equations which describes the retention process.

Furthermore, the separation of total mobility loss and maximum load capacity permits a new and by far less artificial definition of sequestration. The sequestration compartment may now be constituted by those macrophages in the macrophage pool which have a particle load falling in between the load causing total loss of mobility and the maximum load capacity of the macrophages.

To some degree, the new sequestration concept may even be able to account statistically for the chemotactic signals which decaying macrophages may send out; viable macrophages receiving the message and migrating to the location of decay might arrive there prior to the disintegration so that they could incorporate the available debris immediately and leave no time for the released particle clusters to "escape" in part to the interstitium. If the chemotactic signal is assumed to be proportional to the loss of mobility, then the remaining probability for released clusters to lose some of their primary particles to the interstitium would be proportional to the mobility of

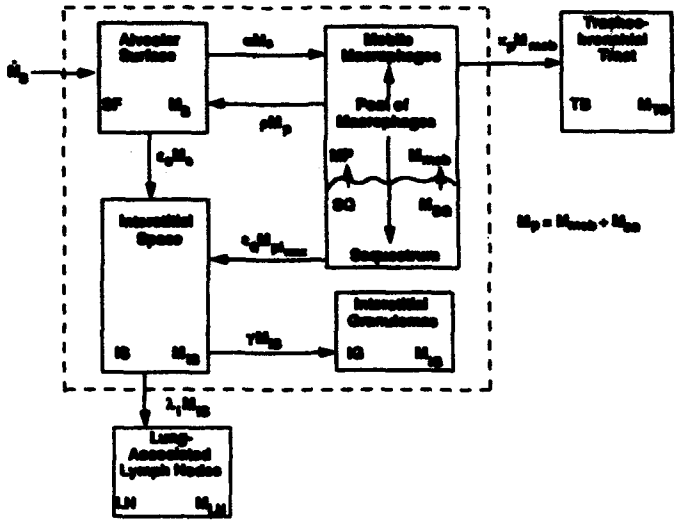

FIGURE 17.

Revised physiology-oriented compartmental kinetics model corresponding to the simplified model shown in Figure 4.The revised model uses the same assignments for compartments and transfer rate coefficients as in Figures 2 and 4. There is no longer a compartment for the decaying macrophages, and the sequestrum is now part of the macrophage pool. The sequestrum of immobile macrophages will now grow on the expenses of the mobile macrophages.

its parent macrophage at the time of release. The transfer of primary particles of the clusters to the interstitium would then have an effective rate coefficient of $\epsilon_{eff} = \epsilon_s f$.

The diagram in Figure 17 shows the essentials of the second generation of the physiology-oriented compartmental kinetics model. The decaying and the sequestered macrophages are now contained in the macrophage pool which consists of a constant total number of more or less viable as well as immobilized macrophages grouped according to whether their particulate load is below or exceeds the volume load limit of mobility, v_{mob}.

This system has a number of modeling options. For exclusive two-partner encounters of macrophages and clusters the rigid irreversibility of sequestered particle mass would be maintained. However, this irreversibility may not persist, if chemotactic migration of the viable macrophages leads to an encounter of several of these with a decaying macrophage which is going to release its particle cluster. In this case, load sharing may occur, and thus, removal of particle mass from the sequestrum by the remaining mobility of the sharing macrophages may be possible.

Another cause for imperfect irreversibility could be that some of the sequestered macrophages might be "grown-over" by the epithelial walls, and thus, their particle content would be relocated in the interstitial space, a process which was envisaged in the original model [see Equation (9)]. We have revived this particular transfer route in Figure 17 and related the rate coefficient, ϵ_Q, to the maximum load capacity volume, v_{max}, of the macrophages.

In a preliminary, first simulation attempt of the experimental data of the selected studies by the revised model, we refrained from accounting for the time dependency of the sequestration onset in the chronic diesel exhaust exposure study at ITRI (Figure 7) and simply used the basic design represented in Figure 17. The details of the mathematical derivation of the system of coupled differential equations and their computerized numerical solution will be given in a separate paper which is presently in preparation.

The Figures 18 to 26 are the equivalents of Figures 8 to 16 and show the quality of the final

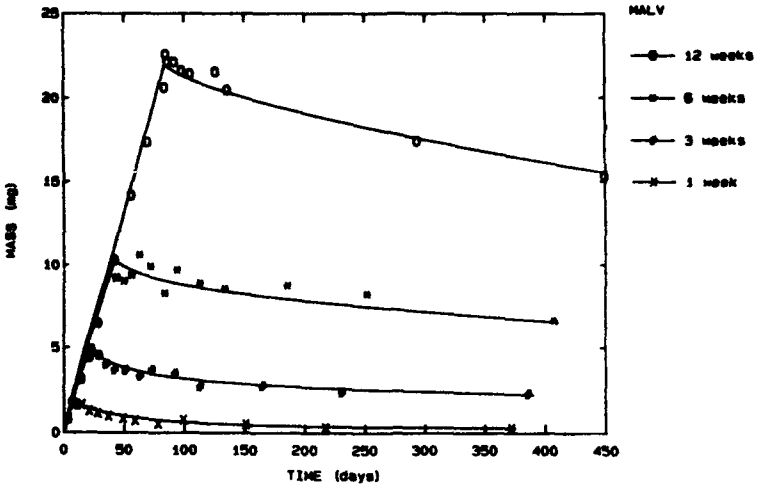

FIGURE 18.

Experimental data of total alveolar retention of diesel soot during and after subchronic diesel exhaust exposures (7 to 84 days) conducted by Strom et al., 1988, and corresponding data simulation curves of the revised retention model.

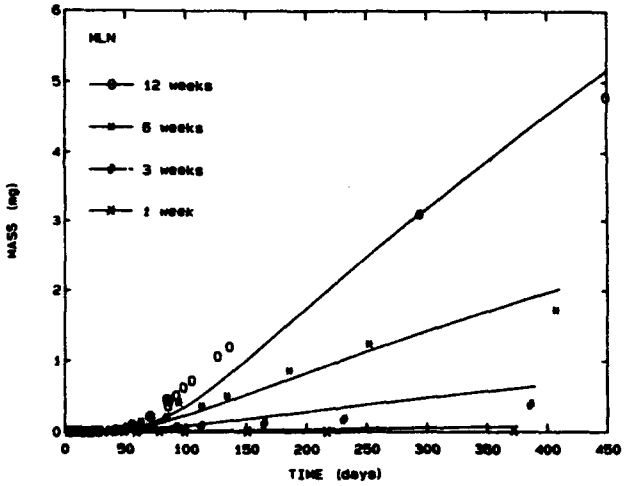

FIGURE 19.

Experimental data of lymph node retention of diesel soot during and after subchronic diesel exhaust exposures (7 to 84 days) conducted by Strom et al., 1988, and corresponding data simulation curves of the revised retention model.

simulations. Similarly, the Tables 5 to 7 correspond to Tables 2 to 4 and give the data and parameters utilized in these simulations.

In case of the alveolar particle mass burden, Figures 18 and 21 show that the simulation quality achieved for the subchronic studies with diesel exhaust and carbon black seems to be as good or somewhat better than obtained with the previous version of the model. For the high concentrations applied in all of these experiments, the lymph node burdens (Fig. 19) show in case of diesel soot a slight overrepresentation for the short exposures, while the longest exposure

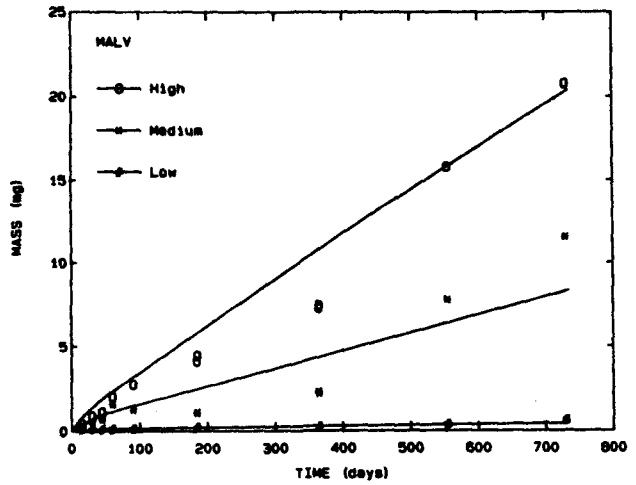

FIGURE 20.

Experimental data of total alveolar retention of diesel soot in chronic diesel exhaust inhalation exposures at ITRI (Wolff et al., 1987), and corresponding data simulation curves of the revised retention model.

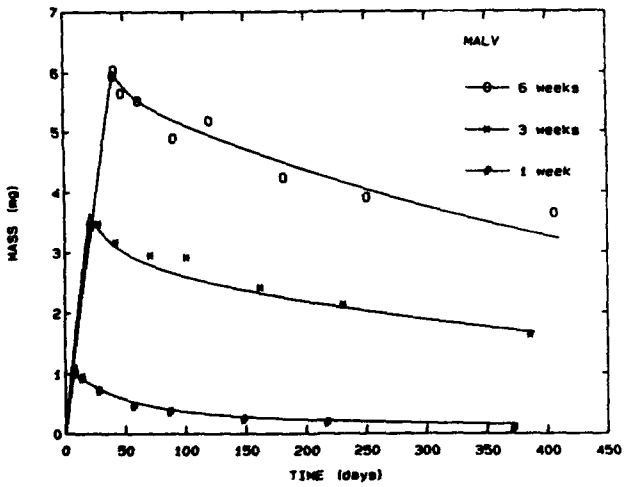

FIGURE 21.

Experimental data of total alveolar retention of carbon black during and after subchronic exposures (7 to 41 days) conducted by Strom et al., 1989, and corresponding data simulation curves of the revised model.

is initially slightly underrepresented for some 200 days. For the lymph node burdens of carbon black (Fig. 22), the latter pattern was even more pronounced in all of the three exposure runs. The corresponding simulations by the previous model show qualitatively the same behavior, but on a smaller scale.

Considering that no time dependent adjustment function was introduced, the chronic diesel exhaust exposure data were reasonably well approximated, although the simulation did not reproduce the final increase in the low exposure experiment. But then, a recent reference to this

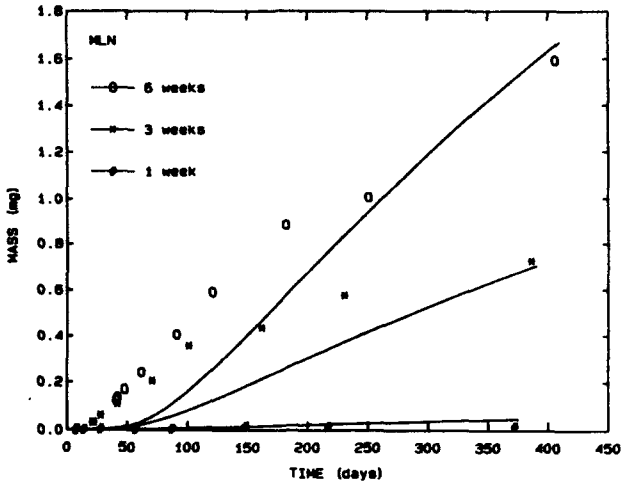

FIGURE 22.

Experimental data of lymph node retention of carbonblack during and after subchronic exposures (7 to 41 days) conducted by Strom et al., 1989, and corresponding data simulation curves of the revised retention model.

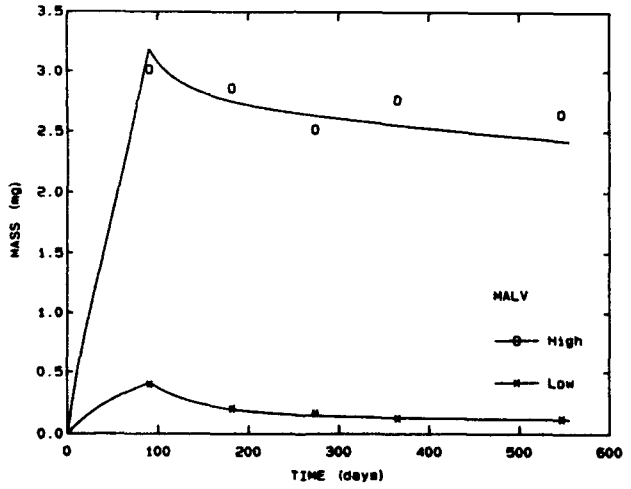

FIGURE 23.

Experimental data of total alveolar retention of test toner during and after subchronic exposures of 90 days as conducted by Muhle et al., 1989, and corresponding data simulation curves of the revised model.

study in an author's review (Snipes, 1989) seemed to assess this deviation as insignificant. Our simulations of the two other exposure runs coarsely approximated the final alveolar load at the end of exposure but, except for the initial phase, they exceeded the experimental values of the first year considerably.

The quality of the new simulation of the test toner data is obviously excellent. This may or may not be due to the fact that, according to the model, the rather coarse size of the toner particles leads to only 17 discrete size classes of macrophage load which, in contrast to the case

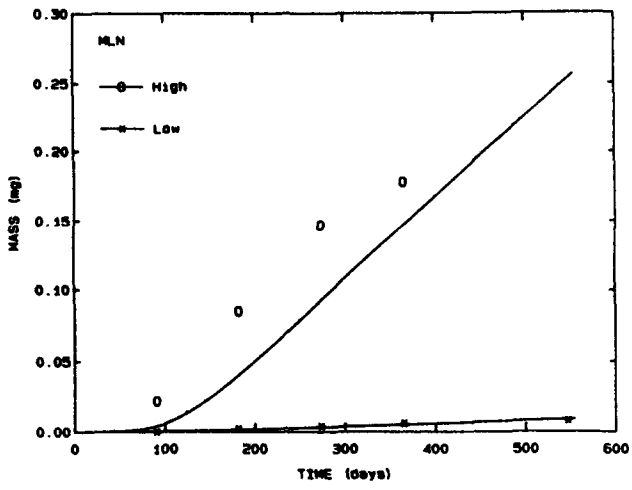

FIGURE 24.

Experimental data of lymph node retention of test toner during and after subchronic exposures of 90 days as conducted by Muhle et al., 1989, and corresponding data simulation curves of the revised retention model.

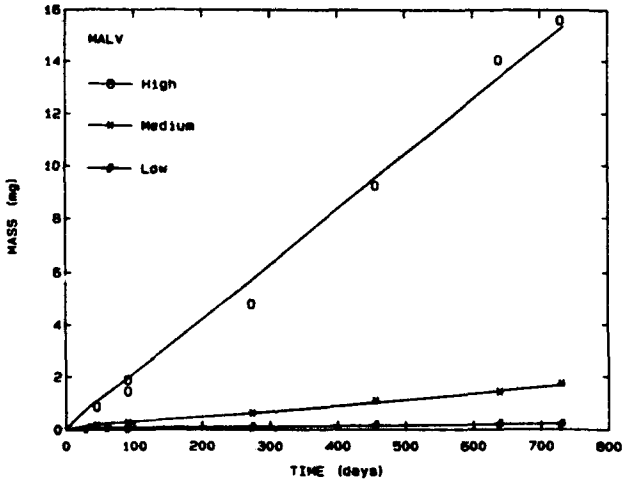

FIGURE 25.

Experimental data of total alveolar retention of test toner in chronic inhalation exposures at Fh-ITA (Muhle et al., 1988), and corresponding data simulation curves of the revised retention model.

of the highly disperse diesel soot and carbon black, can be handled numerically by the computer program without increasing the class intervals.

Comparing the parameters utilized in the two model versions (i.e. Table 2 to 4 vs. Table 5 to 7), the inconsistencies of the previous model are indeed avoided in the new version. The model parameters in Table 5 which are constant for all simulations include now the maximum volume load limit of the macrophages as $v_{max} = 750$ nl. This saves the theoretical foundation of the model and compares well with the estimate by Morrow (1988) of 600 nl. Furthermore, those parameters of Tables 2 and 5 which should be independent of the model version, i.e. τ_L, τ_0, N

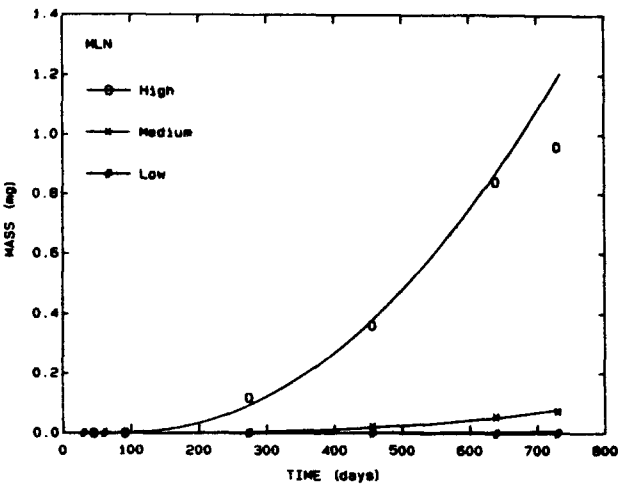

FIGURE 26.

Experimental data of lymph node retention of test toner in chronic inhalation exposures at Fh-ITA (Muhle et al., 1988), and corresponding data simulation curves of the revised retention model.

TABLE 5.

Constant Model Parameters for All Simulations (Revised Model)

τ_L (days)	τ_0 (days)	$\dot{N}$	v_{max} (nl)	κ_{pmax} (day^{-1})	ϵ_s (day^{-1})	ϵ_Q (day^{-1})	γ (day^{-1})
7	0.25	$3.57 * 10^6$	750	0.015	0.03	0.03	0.00

TABLE 6.

Aerosol and Model Parameters Varying with Inhaled Material (Revised Model)

		Diesel Exhaust	Carbon Black	Test Toner
D	(μm)	0.22	0.24	4.0
ρ_D	(g/cm^3)	2	2	1.15
Q_D		0.75	0.75	0.75
v_{mob}	(nl)	750	600	600
v_{crit}	(nl)	720	250	350
λ_i	(day^{-1})	0.0008	0.0020	0.0003

TABLE 7.

Model Parameters Varying with Exposure Conditions (Revised Model)

		Diesel Exhaust Inhalation Strom et al., 1988 Subchronic	Diesel Exhaust Inhalation Wolff et al., 1987 Chronic	Carbon Black Inhalation Strom et al., 1989 Subchronic	Test Toner Inhalation Muhle et al., 1989 Chronic	Test Toner Inhalation Muhle et al., 1989 Subchronic
Exposure Concentrations	(mg/m^3)	6	0.35; 3.5; 7.0	6-7	1; 4; 16	10; 40
Exposure Conditions	$\dot{M}_s$ (mg/day)	0.26; 0.27; 0.30; 0.32	0.0028; 0.028; 0.056	0.152; 0.200; 0.178	0.0014; 0.0057; 0.0315	0.0078; 0.046
	t_{exp} (days)	7; 21; 42; 84	730	7; 21; 41	730	91
	t_{end} (days)	375 - 460	730	375- 415	730	554

resp. $\dot{N}$ and γ, remain indeed unchanged or correspond as $N = \dot{N}\tau_R$. Moreover, the revised model adds now the classical alveolar clearance rate coefficient, κ_{pmax}, to the list in Table 5. Although the corresponding values of the previous model are listed in Table 3, there is good agreement: they show rather little variation and average close to the constant value for the new version.

Finally, the transfer rate coefficients of free particles and of macrophages with maximum particle burdens to the interstitium, ϵ_s and ϵ_Q, respectively, could also be kept constant in all of the new simulations and, thus, are added to Table 5. These latter two coefficients, however, are not unique and occur in Table 5 by choice because there is some degree of freedom due to the lack of quantitative experimental data for the alveolar subcompartments. Within a certain range, different pairs of values ϵ_s and ϵ_Q can be assigned without changing significantly the simulation of the empirical data on M_{ALV} and M_{LN} in the five experimental studies.

With the preceding changes, the only parameter left to be commonly shared in Tables 3 and 6 is the transfer rate coefficient of particulate material from the interstitial space to the lymph nodes, λ_i. Again, there is reasonably close agreement between the corresponding values obtained by best fit for the two model versions. In addition to these values, Table 6 provides size and density data of the inhaled material, which are used in the new model version. Furthermore, as could be expected, the macrophage mobility parameters, v_{mob} and v_{crit}, turned out by best fit to depend upon the material inhaled. Thus, the load volume for vanishing macrophage mobility, v_{mob}, shows a similar behavior as found for M_{pmax} in the previous model (Table 2). Likewise, the critical volume load of the macrophage, v_{crit}, at which the mobility starts to be affected, gave material-dependent values by best fit, as did the analogous values of M_{cf} (Table 2) in the old version.

The fitted values for the mobility limit volume loads, v_{mob}, show unusual patterns: The particles of diesel soot and carbon black are, except for the organic coating on the soot, chemically and physically very similar; their mobility limits, however, are different. For the soot, the loss of mobility, v_{mob}, coincides with the maximum capacity, v_{max}, but the corresponding value for carbon black is 20 % less. On the other hand, carbon black and test toner, although of different bulk density and tremendous size difference, cause the macrophages to lose their mobility at the same volume load value, i.e. at 80 % of v_{max}. For the critical volume, v_{crit}, at which mobility starts to decline gradually, there is no simple pattern either: diesel soot particles seemingly do not impair the mobility of macrophages before they are loaded close to the maximum capacity, while carbon black and test toner particles affect macrophage mobility at relative loads of 33 % (v_{crit} = 250 nl) and 47 % (v_{crit} = 356 nl), respectively. As in the previous model, this is at variance with the estimates by Morrow (1988) who expected a general 10 % load level (60 nl) to affect macrophages.

Comparing Tables 4 and 7, there are no longer model parameters in Table 7 which change with exposure conditions, because the new version does not employ a time function for sequestration. Thus, Table 7 lists only the exposure conditions and the fitted values for the deposition rate, $\dot{M}_s$. These values either match closely the data in Table 4 or, in case of the two subchronic studies on diesel exhaust and carbon black, show a sequence of increasing deposition rates at and above the corresponding data in Table 4. Assuming almost constant respective exposure concentrations, this is probably the previously discussed effect of the increasing ventilation rate in the young rats by their growth during the exposure periods of different duration. The expectable effect may have been obscured in the previous model.

With the results shown here, we think the new model has a reasonably good justification. The unusual and unexplained patterns of the macrophage mobility parameters, v_{mob} and v_{crit}, may need further elucidation by additional experimental results in the future. However, at the present time, the revised model seems to be as consistent as required by the self-imposed criteria. There are still some quantitative deficiencies with regard to the proper representation of the data of the ITRI study, which is the only chronic diesel exhaust inhalation study with lung burden data so far available. Improvements may also be needed with regard to the systematic

deviations of the simulation data from the experimental lymph node burdens in the carbon black study. It may well be possible to correct these flaws by adding other transfer routes to the new model, like those contemplated in Figure 2. However, in the absence of further experimental results with Fischer 344 rats, such an attempt appears to be premature at this time.

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Particle Loading in the Human Lung—Human Experience and Implications for Exposure Limits

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ABSTRACT

Timbrell's analyses of fiber burdens in the post-mortem lungs of workers with long-term inhalation exposures to a variety of amphiboles have shown that the clearance of fibers is strongly dependent on lung burden and its associated lung fibrosis, with a small percentage of very heavily exposed workers having little, if any clearance from parts of the lung. The extent of lung fibrosis is proportional to the total surface of retained mineral particles for both fibers and more compact particles. The human data base from the asbestos workers can provide a sound basis for the development of more generic models describing the influence of lung burden of mineral dust on particle deposition in, and clearance from, human lungs. The implications of the results obtained to the pathogenesis of chronic lung diseases and the evaluation and/or establishment of exposure limits are also discussed, along with some research needs to facilitate interspecies extrapolation of fiber toxicity data.

INTRODUCTION

Our assignment for this Symposium paper was to discuss the following The following issues:

- What evidence, if any, do we have from human epidemiology data that would tend to validate or argue against the rat data with respect to lung overloading?
- What are the implications of lung overloading with respect to environmental health standards?

With respect to the first issue, we have interpreted epidemiology broadly, and will include a review of data from a variety of studies of human lung tissue

Key Words
Particle retention
Fiber burdens
Post-mortem human lungs
Lung fibrosis
Occupational & environmental exposure limits
Critical fiber dimensions
Particle surface

of occupationally exposed workers by one of us (V.T.) in association with numerous colleagues (Timbrell, 1982, 1983, Timbrell et.al., 1988, 1990). Our review of other human exposure-response literature will be brief, since little of it provides quantitative information on lung dust retention in relation to exposure duration or intensity.

The human data base, while limited, shows evidence that overloading of clearance mechanisms is a major determinant of lung fibrosis among workers in the dusty trades. However, it remains inadequate for valid intercomparisons with the results of controlled exposure studies in rats and other laboratory animals. Furthermore, some of the critical determinants of disease potential established in the human studies have not been measured or reported in the results of the animal studies, e.g. particle surface areas, and, for fibers, fiber length and diameter distributions. These critical fiber dimensions differ for the three different diseases associated with asbestos exposure as discussed previously (Lippmann, 1988). The critical fiber dimensions are summarized in Table 1. We will discuss the research needs that we have identified to achieve a more definitive resolution of interspecies differences in response to inhaled mineral dusts.

With respect to the second question, it is already clear that so-called "inert" or "nuisance" dusts can produce adverse effects when clearance mechanisms become overloaded, and that the current occupational exposure limits deserve a careful re-examination. We also review recent evidence that current non-specific ambient air quality standards may not fully protect the more sensitive segments of the population from adverse health effects. This suggests the presence of a threshold for response, analogous to the particle clearance overload phenomemon that has been observed at higher exposure levels in chronic animal exposure studies, as discussed later in this paper.

TABLE 1

Summary of Recommendations on Asbestos Exposure Indices*

Disease	Relevant exposure index
Asbestosis	Surface area of fibers with: Length > 2 μm; diameter > 0.15 μm
Mesothelioma	Number of fibers with: Length > 5 μm; diameter < 0.1 μm
Lung cancer	Number of fibers with: Length > 10 μm; diameter > 0.15 μm

* Reprinted by permission from Lippmann (1988).

Evidence for Dust Overload in Human Epidemiology

Snipes (1989) has estimated that a lung burden of 10 to 20 g in humans corresponds to 10-20 mg particles per gram of wet lung, which is similar to the lung burden of Diesel soot in rats after 24 months of exposure to 3.5 or 7.0 mg soot/m^3. These soot concentrations are clearly "overload" doses in the rat. Thus, evidence of coal workers pneumoconiosis (CWP) among miners who accumulate 10-40 g in British coal mines (Rossiter et al., 1967, Davis et al., 1983, Ruckley et al., 1984, Soutar et al., 1986) or 25 to 50 g in German coal miners (Stöber et al., 1967) is quite consistent with the overload hypothesis. The prevalence of CWP varies with coal rank and other factors for reasons that remain elusive. Data on other potentially important exposure variables, such as particle size distribution and specific surface area are not generally available to separate the effects of mass loading from those associated with surface properties or chemical-specific interactions with epithelial cells.

For community air data, the enhanced response due to overloading may not be comparable to that in the dusty trades. There is however, some epidemiologic

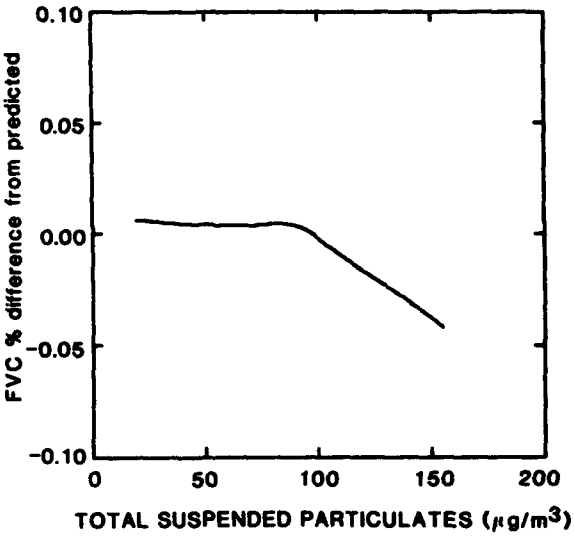

FIGURE 1. The line shown represents a nonparametric fit of the percentage difference between the observed FVC and the FVC predicted by a regression controlling for the effects of standing height, sitting height, age, race, sex, body mass index, smoking, and respiratory conditions smoothed against TSP ($\mu g/m^3$). Reprinted by permission from Schwartz (1989).

evidence for a threshold type of response for total suspended particulates (TSP) in relation to morbidity indices. Schwartz (1989) examined lung function in 4300 representative children and young adults (ages 6-24) in relation to TSP in their communities, using data collected during the second National Health and Nutrition Examination Survey (NHANES II) conducted in the period 1976-1980 throughout the U.S. He found highly significant associations between forced vital capacity (FVC) and peak flow rates and TSP. As shown in Figure 1, there appeared to be a threshold of response at about 90 $\mu g/m^3$ daily average TSP.

In another use of secondary data sources for studies of the health effects of air pollutants, Ostro and Rothschild (1989) regressed data on respiratory-related restricted activity days (RRAD) from the 1976-81 Health Interview Survey (HIS) against the daily average concentration of fine particulate matter (FP), i.e., mass concentration of aerosol $\leq$ 2.5 μm in aerodynamic diameter. The HIS is a national (U.S.) multistage probability survey of working individuals, aged 18-65 in 50,000 households. Respondents were asked to report RRAD in the prior 2-week period, such as days of work loss or bed disability as well as more minor restrictions associated with an acute respiratory condition. They found the best estimate for the effect of FP on RRAD to be a 1.58% increase for each 1 $\mu g/m^3$ of FP.

Ozkaynak and Thurston (1987) reported on associations between 1980 U.S. mortality rates in 98 Standard Metropolitan Statistical Areas (SMSAs) and four measures of particulate air pollution. These were total suspended particulate matter (TSP); inhalable particulate matter, i.e., particulate < 15 μm in aerodynamic median diameter (IHP); fine particulate matter, i.e., particulate < 2.5 μm in aerodynamic median diameter (FP); and sulfate (SO^{2-}_4), a major component of FP. They found that FP and SO^{2-}_4 were most consistently and significantly associated with the reported SMSA-specific total annual mortality rates, whereas TSP and IP were often nonsignificant predictors of mortality.

The analyses of Ostro and Rothschild (1989) and Ozkaynak and Thurston (1987) did not consider a threshold model. Furthermore, their results suggest that the FP associated responses are due to the acidic nature of the small particle fraction and not the mineral dust in the coarse particles that dominate the IHP and TSP measures. Thus, the threshold type of response reported by Schwartz (1989)

for TSP and lung function may, or may not, apply to RRAD and daily mortality. In any case, it is uncertain how the community air pollution results relate, if they do, to the overload hypothesis based on chronic exposures at much higher concentrations.

Evidence for Dust Overload in Human Lung Studies

Timbrell (1982) developed a model for fiber deposition in human lungs based upon his analysis of the bivariate diameter and length distributions found in air and lung samples collected at an anthophyllite mine at Paakkila in Finland. At this particular mine, the length and diameter distributions of the airborne dust were exceptionally broad, and historic exposures were very high. He observed that, for workers with the highest exposure and most severe lung fibrosis (Ashcroft et al., 1988), the lung fiber distributions in some tissue segments approached those of the airborne fibers. Adjacent tissue, analyzed for extent of fibrosis, showed severe fibrotic lesions. He concluded that long-term retention was essentially equal to deposition in such segments, and that the fibrosis in the tissue had not affected deposition. His deposition model, illustrated in Figure 2, is based upon the bivariate size distribution differences between airborne dust samples and the dust in the most heavily fibrosed lung tissue. Figure 3 shows a series of retention curves for different degrees of lung fibrosis. These curves were determined by comparing the fiber size distributions in other tissue samples from the same lung with the distribution in the sample for which all fibers deposited were retained.

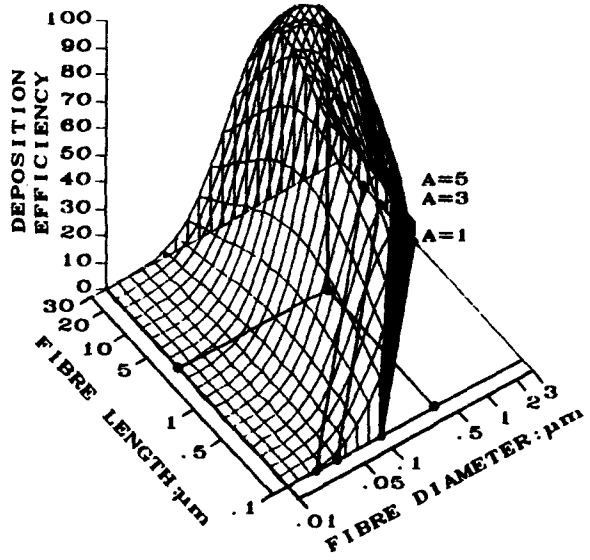

FIGURE 2. Bivariate plot of deposition efficiency model for the gas-exchange region of the human lung as a function of fiber length and fiber diameter. Limits are shown for aspect ratios (A) equal to 1, 3, and 5. The model is based on differences between airborne fiber distributions and distributions measured in very severely fibrosed lung tissue.

The deposition model was tested by comparing the fiber retention found in the lungs of much less heavily exposed asbestos workers at Paakkila and other locations to that predicted by applying the deposition model to the specific airborne dust distributions. Figure 4 shows the bivariate size distributions for airborne fibers at the Transvaal in South Africa (crocidolite and amosite), at the Northwest Cape in South Africa (crocidolite) and at Wittenoom in Australia

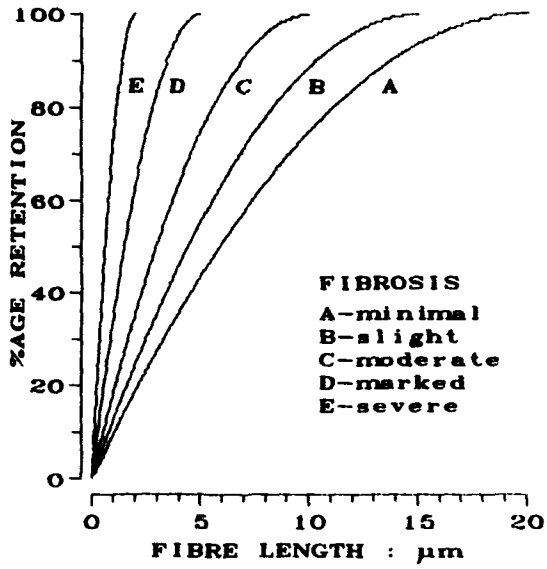

FIGURE 3. Effect of lung fibrosis on fiber retention in human lungs as a function of fiber length. The model is based on retention in lung segments from the same lung used to develop the model illustrated in Figure 2, but with various lesser degrees of fibrosis.

(crocidolite). Figure 4 also shows the distributions of fiber lengths for specific fiber diameter intervals. This is a particularly useful form of presentation for studies of the influence of fiber diameter on mesothelioma. It is clear that as one goes from Paakkila to the Transvaal, to the NW Cape, to Wittenoom, both the lengths and diameters shift downward substantially.

Figure 5 shows the predicted bivariate size distributions based on combining the data used to develop Figure 2 with the data from Figure 4 for workers at Paakkila and Transvaal, with relatively light occupational exposures, as well as the observed bivariate lung distribution data from these regions. It is apparent that the model fits the observations satisfactorily.

Figures 6 and 7 show the influence of fiber size and degree of lung fibrosis on fiber retention and clearance for the fiber size extremes represented by Paakkila and Wittenoom respectively. Paakkila, with virtually no fibers with diameters less than 0.1 µm, produced many cases of lung cancer and asbestosis, but no mesothelioma. Wittenoom, with virtually all of the fibers having diameters less than 0.1µm and lengths less than 5µm, produced a very high yield of mesothelioma and lung fibrosis, as well as an excess in lung cancer (SMR=1.57) (Hobbs, et al., 1980). These figures also show the great differences in the fibers retained in and cleared from the gas exchange region of the lung at Paakkila and Wittenoom. The cleared fibers represent the longest capable of reaching the pleural surfaces where mesotheliomas are found. The modal values for both fiber length and diameter are also shown in Figures 6 and 7. The differences between retained and cleared fibers' modes are much greater for both the minimal and severe fibrosis cases for the longer, thicker fibers at Paakkila than for the shorter, thinner fibers at Wittenoom. The data indicate the need to review the critical dimensions of fibers for mesothelioma production. Long fibers may be more carcinogenic than shorter fibers. The fibers at Wittenoom were almost all shorter than five µm, but were present in enormous numbers. In fact, Rogers (1990) reported on reanalyses of thermal precipitator slides from Wittenoom using currently used analytical techniques, and reported about 300 fibers/mL greater than 5 µm in length.

Lung fibrosis is associated with increased fiber retention, and fiber retention is clearly associated with fiber length and diameter. More precise descrip-

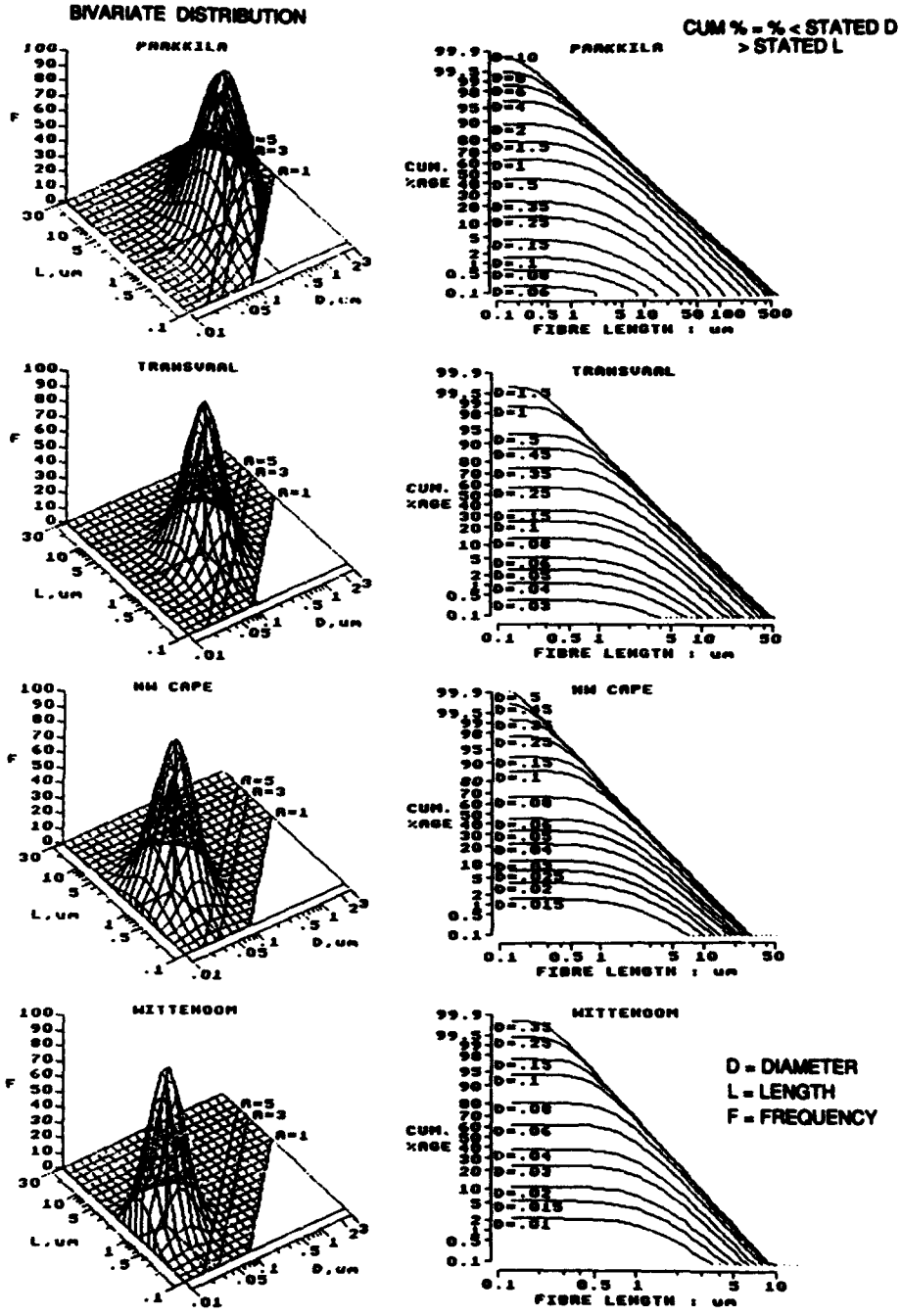

FIGURE 4. Size distributions of airborne fibers collected at asbestos mines and mills at Paakkila, Finland, The Transvaal and Northwest Cape in South Africa, and at Wittenoom in Australia. The left side shows bivariate frequency distributions by length and diameter. The right side shows the cumulative percentages less than the stated diameter that are longer than the indicated length.

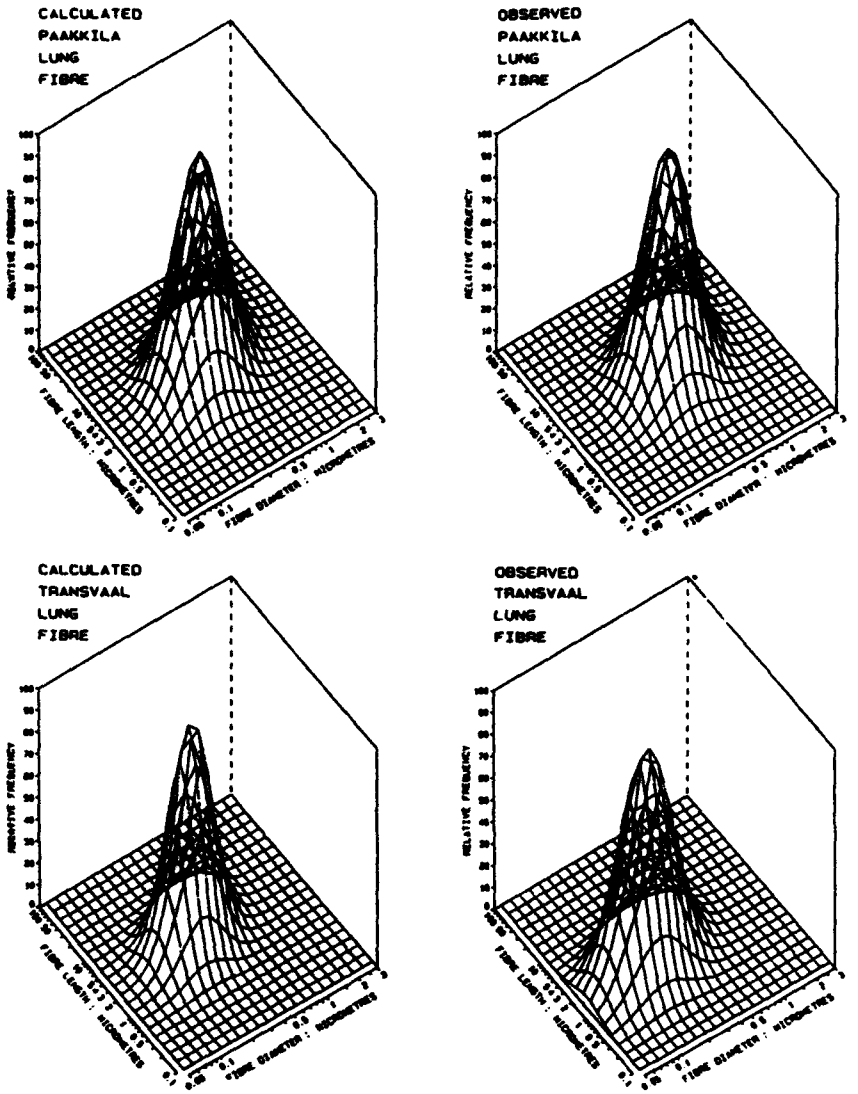

FIGURE 5. The left side shows bivariate fiber length and diameter distributions calculated for retention in Paakkila and Transvaal miners' lungs based upon Timbrell's deposition and retention model as applied to measured airborne fiber distributions. The right side shows the bivariate size distributions measured in miners' lungs at these locations.

tions of the effect of fiber loading in the lung on fibrosis need to be based on the use of the most appropriate index of fiber loading. Figure 8 clearly shows that the only fiber concentration index that normalizes the diverse data from the various asbestos mining regions is the total fiber surface of the aerosol. When fiber number concentration or total fiber mass concentration is used, each mining region exhibits a quite different exposure-response relationship.

Timbrell (1989) next asked the question of whether the clear association between particle surface concentration and lung fibrosis was limited to fibers. Lung samples were collected from 39 dust exposed workers from a variety of locations including gold mines, shipyards, etc. and bivariate size distributions were analyzed from tissue adjacent to that used to determine the extent of lung

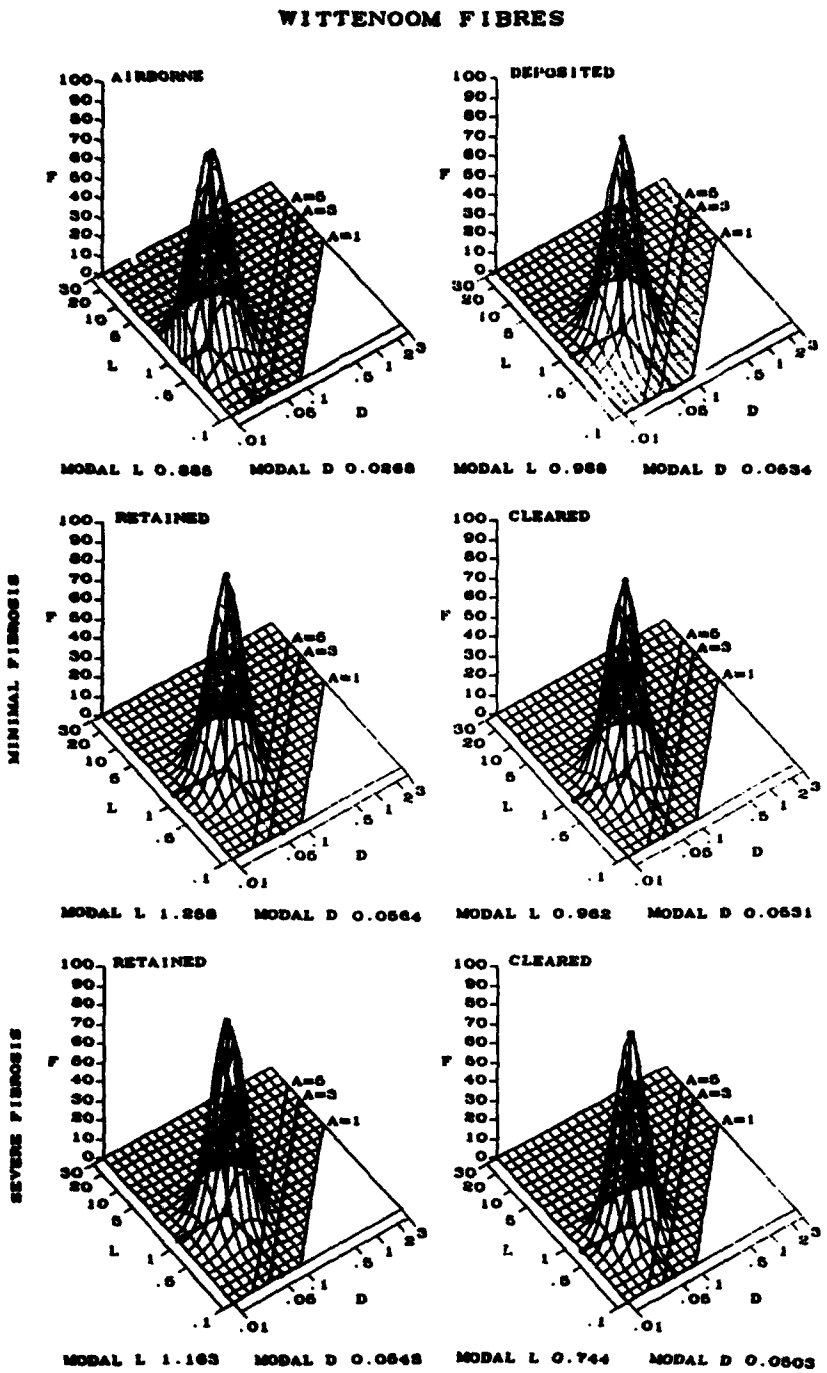

FIGURE 6. Bivariate distributions of asbestos fibers at Wittenoom that were: 1) airborne; 2) deposited in the deep lung; 3) retained in a part of the lung with minimal fibrosis; 4) cleared from lung tissue with minimal fibrosis; 5) retained in a part of the lung with severe fibrosis; and 6) cleared from lung tissue with severe fibrosis.

PAAKKILA FIBRES

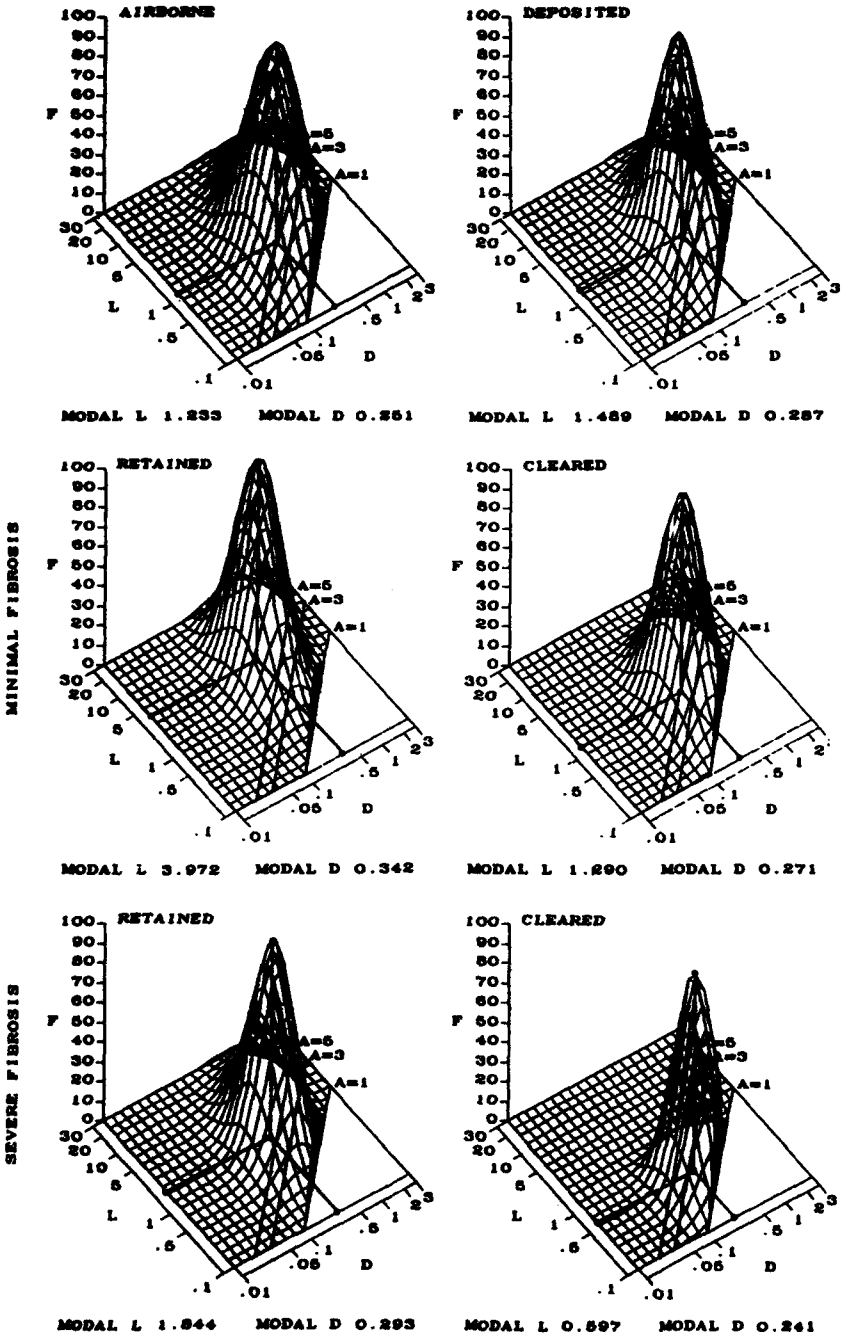

FIGURE 7. Bivariate distributions of asbestos fibers at Paakkila that were: 1) airborne; 2) deposited in the deep lung; 3) retained in a part of the lung with minimal fibrosis; 4) cleared from lung tissue with minimal fibrosis; 5) retained in a part of the lung with severe fibrosis; and 6) cleared from lung tissue with severe fibrosis.

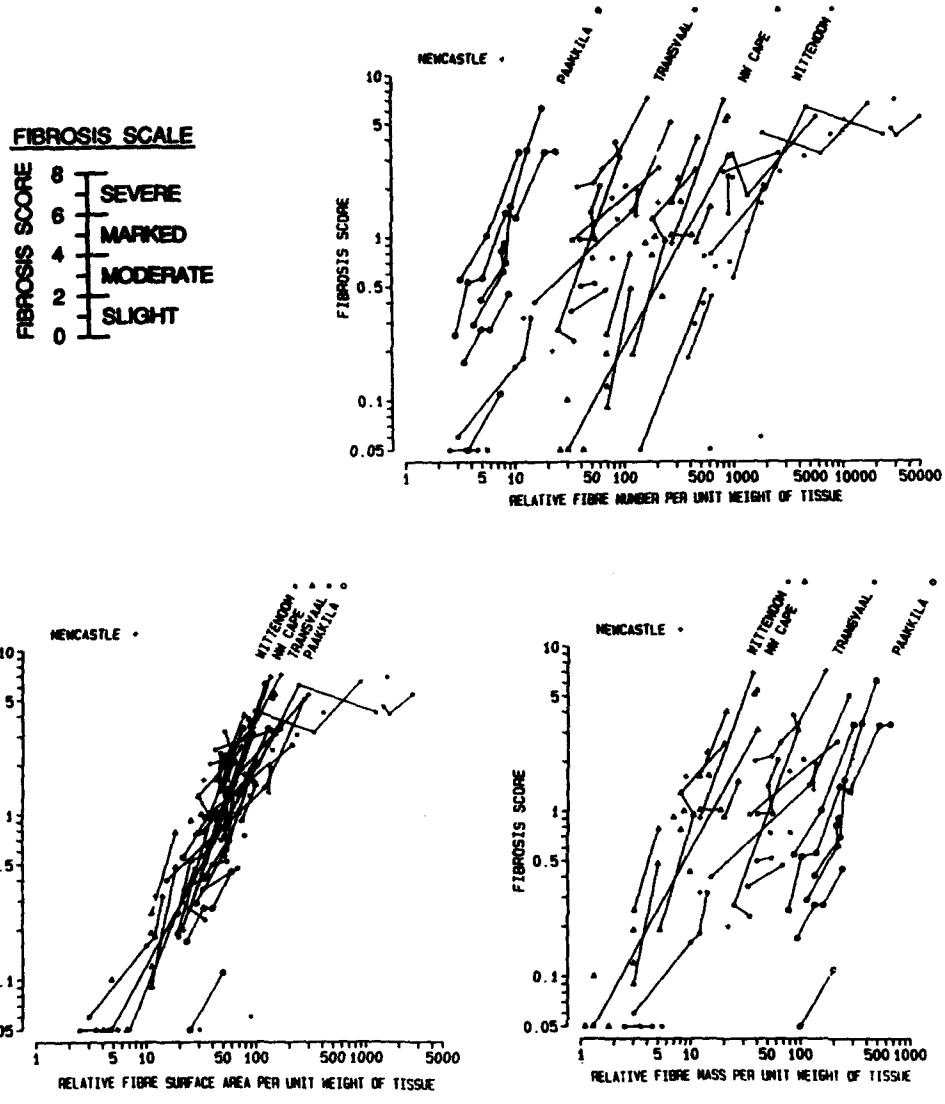

FIGURE 8. Relationships between fibrosis scale and relative concentrations of fibers per unit weight of dry lung tissue. The lines connect data points from the same subject. The relative fiber surface area normalizes the data better than either the relative fiber number concentration or the fiber mass concentration. Reprinted by permission from Lippmann (1988).

fibrosis. As shown in Figure 9, the correlation between dust surface area and the degree of fibrosis was much better (r=0.80) than for particle number concentration (r=0.50). He then analyzed whether different components of the dust mixtures in these lungs contributed disproportionately to the fibrotic response. The results are summarized in Table 2 in terms of the asbestos alone, the asbestos plus quartz, and the other constituents generally considered much less fibrogenic than asbestos or quartz. The fibrogenicity coefficient of 2.38 units for the category "other then asbestos and quartz" is not substantially different from those for the more "fibrogenic" dusts, suggesting that its components, including talc, mica, various other silicates and iron are as fibrogenic as asbestos and quartz when expressed in terms of particle surface area. The table also shows

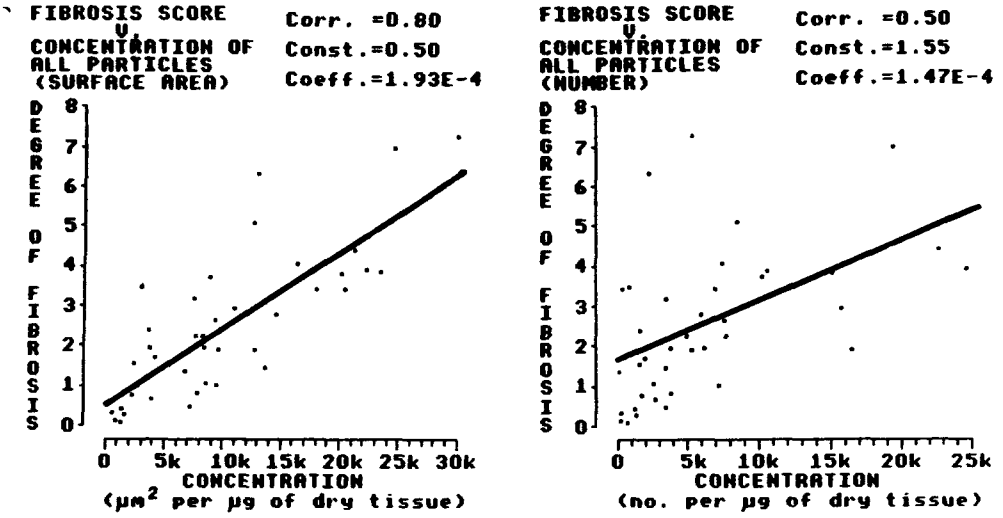

FIGURE 9. The left panel shows the relationship between fibrosis scale and concentrations of total particulate surface per unit weight of dry lung tissue for workers in a variety of dusty trades. The right panel shows the results for the same tissue samples when expressed as particle number concentration.

that concentrations expressed as particle volume had correlation coefficients somewhat lower than those for particle surface, but much higher than those for particle number concentration. Thus, respirable mass concentrations, as conventionally measured for dust exposed workers, are better surrogate measures of fibrogenicity hazard than particle counts for the dusty occupations represented in this limited study.

TABLE 2

Fibrogenicity (and Correlation) Coefficients for Fibrosis Score vs. Particle Concentration for 39 Lung Samples of Workers with Various Mineral Dust Exposures*

Types of particles included in analysis	Concentration index+ Surface	Volume	Number
All measured particles	1.93[a] (0.80)	1.26[b] (0.69)	1.47[c] (0.50)
Asbestos only	2.70[a] (0.72)	2.07[b] (0.64)	2.22[c] (0.49)
Asbestos & quartz	2.03[a] (0.71)	1.57[b] (0.65)	1.47[c] (0.49)
Other than asbestos & quartz†	2.38[a] (0.43)	1.43[b] (0.44)	2.65[c] (0.33)

* Specimens obtained from long-term workers at asbestos mines and factories, gold mines, a platinum mine, British shipyards and other work.

\+ Derived from high resolution transmission electron microscopy by Finnish Acad. Sci.

† Includes talc, mica, kaolinite, iron, etc.

a Degree of fibrosis/μm^2/μg dry tissue X 10^{-4}.

b Degree of fibrosis/μm^3/μg dry tissue X 10^{-4}.

c Degree of fibrosis/no./μg dry tissue X 10^{-4}.

Note: Comparison across rows is invalid for fibrogenicity coefficients, since the fibrogenicity units differ.

IMPLICATIONS FOR EXPOSURE LIMITS

Overloading of particles in the lung can be operationally defined as a marked reduction in lung clearance, and the evidence is clear from both the animal and human studies reviewed at this Symposium that such reductions in clearance are threshold phenomena. If we had definable thresholds it would be relatively easy to set occupational and environmental exposure limits for airborne particles. We would need only define the particle properties to be measured and the exposure-response relationships for the health endpoints of interest. It would not be necessary to wait for a full mechanistic understanding at the cellular and molecular level. In any case, based upon the presentations and discussions at this Symposium, such an understanding is not likely to be available in the near future.

Furthermore, for the specific case of "insoluble" particles that deposit in lung airways and airspaces, it is not at all clear that the fascinating and complex biochemical events occurring at the cellular and molecular level are as important as determinants of disease potential as the biophysical processes that determine deposition patterns, translocation pathways, and retention times at critical target sites. Perhaps this Symposium needed one more half-day session devoted to issues such as: 1) the implications of the highly concentrated surface deposition at airway bifurcations in both large and small airways, and the virtual absence of deposition in the peripheral alveoli; 2) the influence of particle dimensions on clearance rates and pathways, e.g., the relatively rapid migration of ultrafine particles and fibers with diameters <0.1 μm into the interstitium, and the lack of migration of fibers longer than ~ 10 μm from their initial deposition sites; 3) the critical role of the total surface area of retained particles in the formation of fibrotic lesions; and 4) the relative unimportance of chemical composition in the fibrotic response to durable mineral particles retained in the lungs.

While we know how, in principle, to approach the setting of exposure standards for insoluble mineral particles, it does not follow that we have all of the data we need to set good standards. We will need to more firmly establish which particle properties are to be measured, and how they are to be analyzed. We also need to establish and/or verify that we can relate the defined exposure parameters to the health outcomes measured in the human studies and/or extrapolated from the animal studies. These tasks are formidable, but not impossible. The critical first step is to get the interested parties in the research community and the regulatory authorities to commit the necessary resources to this undertaking.

RESEARCH NEEDS

The lack of biophysical perspectives has inhibited the effective integration of the results of the fairly extensive data bases from the animal and human studies. We strongly suspect that the apparent differences in the toxicity rankings of the various asbestos minerals between the animal and human studies would disappear if the data were adjusted for the different lengths and diameter distributions of the inhaled fibers. As demonstrated by the recent work of Davis et al (1986, 1988) with long and short amosite and chrysotile, the UICC reference samples were much less toxic than the raw materials that many of the mine and mill workers exposed to. The grinding and blending processes used to make the UICC reference materials uniform made them much less suitable for realistic toxicity testing.

To the extent that the bivariate fiber size distributions of the test materials used in the animal toxicity studies can be determined retrospectively, it may be possible to reanalyze the exposure-response relationships from these studies and gain valuable new perspectives. It would also be extremely desirable to do bivariate fiber size distribution analyses on the dust retained in the lungs of the animals that were chronically exposed to durable fibers, and to compare the retention to the degree of fibrosis. This would permit a valid interspecies comparison with Timbrell's data on the lungs of chronically exposed asbestos workers.

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Particle Overload in Toxicological Studies: Friend or Foe?

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ABSTRACT

The overloading of particle clearance is an important issue in the design and interpretation of inhalation toxicological studies. This issue is particularly important in chronic inhalation bioassays in rats, in which overloading is associated with inflammation, epithelial proliferation, and fibrosis, which may amplify carcinogenic responses or, as suggested by some, even induce cancer regardless of the inhaled material. At present, the key issue is whether or not data from exposures causing overload in animals are useful for predicting health effects in man. A review of reports of chronic inhalation studies in rats exposed to a spectrum of materials suggests that not all exposures resulting in overloading cause cancer, and that the cancer incidences from exposures causing overloading appear to reflect the relative carcinogenic potentials of the test materials. Data from such exposures, however, do little to establish the exposure-response relationship at lower doses most typically relevant to human exposures. Responses observed under overload conditions may be relevant to responses of humans exposed to high (occupational) levels of dusts, of humans with clearance impairments, or of humans in whom inflammation, epithelial proliferation, or fibrosis are concurrently induced by other agents. We need to know more about the relative contributions of carrier particles and particle-borne carcinogens to carcinogenicity under overloaded and non-overloaded conditions. We need to know more about the function of retained particles as reservoirs of particle-borne carcinogens. We need to know more about the potential amplification of carcinogenic responses at low doses by clearance impairments and inflammatory, proliferative, and fibrotic responses induced by other agents. Most importantly, we need a better understanding of the mechanisms of carcinogenesis. At this time, we have sufficient ability to design animal studies to either include or avoid overload. It is concluded that it may be useful to include at least a "minimal overload" level in inhalation bioassays of poorly-soluble particles, and that this approach might be a useful substitute for the classical maximum tolerated dose in setting exposure limits.

Key Words: Inhalation Toxicology, Lung Cancer, Particles, Pulmonary Clearance, Particle Overload, Rats, Bioassays, Diesel Exhaust, Dusts, Pneumoconiosis

BACKGROUND

The term, "dust (or particle) overload," has been coined for the condition in which the lungs are exposed repeatedly to inhaled poorly-soluble particles at rates which cause an overwhelming of particle clearance defenses, leading to a slowing of clearance and the progressive accumulation of particles in the lung in amounts in excess to those which would accumulate in the presence of normal clearance (Morrow, 1988). With chronic exposure of rats to high concentrations of particles, overloading is typically associated with persistent active inflammation, increased epithelial cell proliferation, and fibrosis in the lung, features constituting a "pneumoconiosis" not unlike dust pneumoconioses in man. The term was first used in the literature by Bolton et al. (1983) in reference to the clearance of amosite asbestos fibers from lungs of rats.

The current attention to this phenomenon resulted largely from the carcinogenicity observed in rats exposed to high concentrations of diesel exhaust (Mauderly et al., 1990). Vostal (1986) first called attention to the fact that significant elevations of lung tumor incidences occurred only in rats exposed to diesel exhaust under overloading conditions, and suggested that the tumor response of rats to diesel soot and other "noncarcinogenic dusts" might be a nonspecific, "epigenetic" effect that was not dependent on the genotoxic properties of the particles. Because inhalation exposures of humans to diesel exhaust at concentrations causing overloading and significant carcinogenicity in rats were not expected, the usefulness of the rat carcinogenicity data for predicting potential exhaust-induced lung carcinogenicity in man was questioned.

The relevance of information from studies of inhaled materials in rats exposed under overloading conditions remains in question. The importance of considering this issue in the design of chronic inhalation studies has been discussed (Morrow and Mermelstein, 1987; Lewis et al., 1989; Ames and Gold, 1990), although no firm guidelines have been developed for setting the upper bounds of exposure concentrations for inhaled particles. A group convened by the Toxicology Design Review Committee of the National Toxicology Program recommended that the highest concentration "should produce only minimal interference with the lung defense mechanisms as judged by impaired particle clearance" (Lewis et al., 1989). This group further stated that, for particles less than 3 μm mass median aerodynamic diameter (MMAD), chronic exposures at greater than 100 mg/m^3 are of "limited utility" in assessing risks to the health of humans exposed at lower concentrations.

The dust overloading phenomenon, particularly as it occurs in rats, has been studied from several viewpoints, and is generally agreed to be closely linked to the clearance function of alveolar macrophages (Snipes, 1989). Chan et al. (1984) noted that the rate of alveolar clearance of diesel soot was related to the total amount of soot in the lungs ("lung burden") of rats. They found that repeated exposures of rats to 6 mg soot/m^3, which resulted in a lung burden of 6.5 mg soot, slowed clearance significantly, while no clearance impairment was found at lung burdens less than 0.8 mg. Morrow (1988) suggested a volume-dependent effect of particles on the activity of alveolar macrophages, proposing that macrophage activity ceases when the volume of collected particulate reaches approximately 600 μm^3/cell. Muhle et al. (1988) proposed that overloading was primarily a function of the rate of particle deposition (exposure intensity), and used data from exposures of rats and hamsters to several dusts to suggest that clearance from the deep lung was retarded by chronic exposures to concentrations ranging from 3 to 14 mg/m^3. They also reported that only the particulate, and not the gaseous components, were responsible for delayed clearance induced by combustion emissions. The factors determining the relationships among exposure, clearance, retention, and sequestration of particles continue to be studied and models for predicting the rates of these phenomena have been developed and continue to be refined. Chronic inhalation studies are also being conducted at this time to determine the relative contributions of carrier particles and particle-associated organic carcinogens to the pulmonary carcinogenicity in rats caused by chronic exposure to diesel exhaust.

PURPOSE

The purpose of this report is to consider the usefulness of carcinogenicity data from chronic inhalation studies of rats, which include exposure levels causing dust overloading. The working hypothesis is that such data are useful if they meet one or more of the following criteria: 1) give information on the carcinogenic potential of the test material relative to those of other materials; 2) are produced by exposures that might occur in humans, or which produce lung burdens similar to those which might occur in humans; or 3) are produced under conditions causing lung responses that might occur in humans in which increased particle retention, inflammation, epithelial proliferation, or fibrosis might be induced concurrently by other agents. The approach taken is to use published exposure-tumor response information from chronic inhalation studies of rats exposed to a variety of respirable particles having relatively low solubility. The potential carcinogenicity of diesel exhaust is compared to the carcinogenicities of other materials as a "case study" example. It is recognized that additional information is either now, or will soon be, available from rat studies that are completed but not yet reported, and others that are still under way. Although these studies will not be mentioned in this review, information available at the time of this writing suggests that the new data will be consistent with the conclusions presented herein.

It should be understood at the outset that this report does not attempt a thorough discussion of the variables and confounding factors which would have to be considered if the comparisons made in this report were to be examined rigorously. It is not intended to rigorously analyze the relative carcinogenicities of the materials discussed, or to predict accurately the retention of particles in human lungs. The purpose is to consider the general question of whether or not animal studies using overloading exposure regimes are useful for hazard evaluation. The animal studies compared are diverse in design, analysis, and style of reporting. Considerable liberties are necessarily taken to develop unifying descriptions of exposures and tumor results, in order to make broad comparisons among data sets. Similarly, substantial assumptions and generalizations are involved in the comparisons of potential accumulations of particles in human and animal lungs. This report presents a view of the overloading issue that the author believes should be considered, along with the mechanisms of carcinogenesis, interspecies differences, and other difficulties in dose-response extrapolation, in evaluating current approaches to the assessment of carcinogenic risks. This largely one-sided view supports the inclusion of a minimum of one overloading exposure regime in most bioassays of the carcinogenic hazards of poorly-soluble respirable particles.

USEFULNESS OF STUDIES INCLUDING DUST OVERLOADING FOR EVALUATING THE RELATIVE CARCINOGENIC POTENTIALS OF PARTICLES

Carcinogenicity of Diesel Exhaust

To use diesel exhaust as a case study, the carcinogenicity results from several chronic studies in rats were first synthesized into a single exposure rate-tumor response relationship. The published studies of rats exposed chronically to diesel exhaust were recently reviewed (Mauderly et al., 1990). It has been shown that long-term exposures are required to demonstrate diesel exhaust-induced carcinogenicity in rats; few tumors are observed before 18 months of exposure. The present comparison, therefore, includes only studies in which substantial numbers of rats (approximately 100 or more) were exposed for 24 months or longer (Table 1). Because the studies were of similarly long durations but used different weekly exposure schedules, the weekly exposure rate ($mg \cdot hr \cdot m^{-3}/wk$) was adopted as a useful unifying exposure term. To facilitate the subsequent comparison with exposures to other materials having differing particle sizes (and thus differing fractional pulmonary depositions), all particle concentrations are converted to the approximate American Conference of Governmental Industrial Hygienists (ACGIH) "respirable"

TABLE 1
Pulmonary Carcinogenicity In Rats Exposed
Chronically To Diesel Exhaust

Laboratory (Reference)		Exposure Rate ($mg \cdot hr \cdot m^{-3}/wk$)	Lung Tumor Incidence (%)
Battelle-Geneva		0	1.4
(Brightwell, et al., 1986)		49	0.7
		153	9.7[a]
		459	38.5[a]
Fraunhofer Inst.		0	0
(Heinrich et al., 1986)		347	15.8[a]
Japan Automobile Research Inst.	(light-duty)	0	3.3
(Ishihara, 1988)		9	2.4
		33	0.8
		92	4.1
		192	2.4
	(heavy-duty)	0	0.8
		42	0.8
		84	0
		151	3.3
		309	6.5[a]
Inhalation Toxicology Res. Inst. (ITRI)		0	0.9
(Mauderly et al., 1987)		10	1.3
		107	3.6[a]
		107	6.5[a],[b]
		217	12.8[a]
Natl. Inst. Occup. Safety & Health (NIOSH)		0	3.3
(Lewis et al., 1986)		59	3.8

[a]Difference from control incidence reported to be statistically significant.
[b]From Mauderly et al., 1986.

concentrations using reported information on particle size and graphic analysis (Hinds, 1982). This crude normalization provides a first approximation of a standardized "dosing" term. The diesel soot from all studies was considered to have size characteristics identical to that in the Mauderly et al. (1987) study, as described by Cheng et al. (1984). Because the statistical significances of the exhaust-induced increases in lung tumor incidences were reported for all studies, the exposures reported to cause significant increases are indicated in the table.

The data in Table 1 are plotted graphically in Figure 1. Modeling of the exposure-response relationships for diesel exhaust-induced pulmonary carcinogenicity in rats has been attempted by several groups using different methods and efforts to refine these models are continuing at present. A crude approximation of the exposure rate-lung tumor response relationship was considered adequate for the present purpose; thus, a 2-degree polynomial function was fit to the data to develop the curve shown in Figure 1.

Figure 1 illustrates that the lung tumor incidence (as crudely modeled) of exhaust-exposed rats exceeded the upper limit of the incidence among control rats at an exposure rate of approximately 150 $mg \cdot hr \cdot m^{-3}/wk$. The lowest exposure rate causing a statistically significant increase in tumor incidence was 107 $mg \cdot hr \cdot m^{-3}/wk$ in the Mauderly et al. (1987) study. Because this increase appears to be at the approximate lower limit of detection in studies with practical group sizes (220/group in this case), it is particularly noteworthy that the finding was reproduced in a later study at the same laboratory using the same exposure system and pattern (Mauderly et al., 1986). The second study produced a slightly higher lung tumor incidence (6.5% vs 3.6%) in exposed rats and a slightly lower incidence (0% vs 0.9%) among controls than the first. This

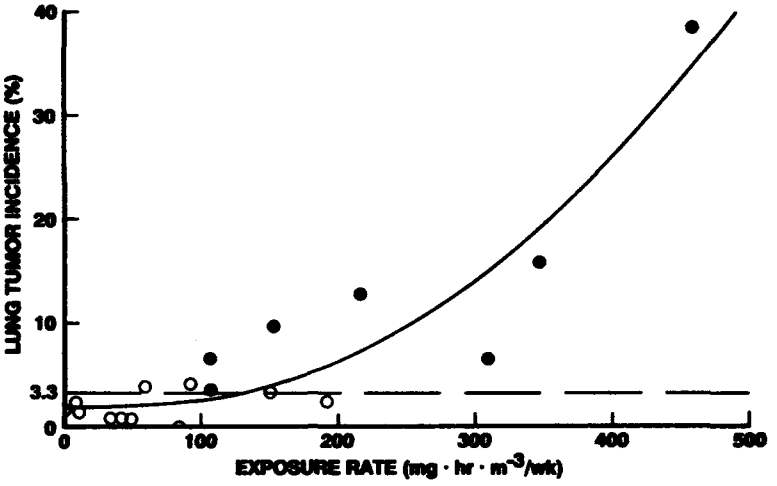

FIGURE 1. Exposure Rate-Lung Tumor Relationship For Diesel Exhaust-Induced Lung Tumors in Rats. A 2-degree polynomial function was fit to the data in Table 1. Solid circles indicate lung tumor incidences reported as significantly higher that those of controls; open circles represent incidences not significantly different from those of controls. The dashed line represents the upper limit of the control lung tumor incidence (3.3%) in any study.

demonstrates the degree of reproducibility of tumor response that can be expected from study to study using closely replicated conditions. In both studies, this exposure rate caused an accumulation of soot in the lung, chronic active inflammation, epithelial proliferation, and focal fibrosis, the hallmarks of dust overload. Two exposures in the Japanese study (Ishihara et al., 1988), at rates of 151 and 192 mg·hr·m^{-3}/wk, did not produce significant increases in lung tumors, even though these exposures produced the histological signs of overloading. Regardless, it is clear from Figure 1, as previously reported, that chronic inhalation of high concentrations of diesel exhaust causes an exposure-related increase in the lung tumor incidence of rats.

Carcinogenicity of Diesel Exhaust Relative to Those of Other Materials

The next step was to compare the exposure-lung tumor response relationship of diesel exhaust to those of other inhaled particles for which similar data exist. Seven materials representing a spectrum of physical and chemical forms were chosen for the comparison (Table 2). The requirements were that each relatively insoluble, respirable material must have been studied by chronic (approximately two years or more) inhalation bioassay using substantial numbers of rats, that more than one exposure level of the test material must have been used, and that the lung tumor incidences must have been reported for each control and exposed group. As stated above, the exposure concentrations for all materials were adjusted to approximate the ACGIH respirable particulate level in an attempt to reduce fractional deposition as a confounding factor.

Oil shale dust represents a mineral dust having approximately a 10% quartz content, and little, if any, bioavailable organic content. The information on raw and retorted oil shale dust was considered acceptable because, although two laboratories conducted the studies using one exposure level each, the exposure materials were identical. The shale dusts for both the ITRI and Los Alamos National Laboratory studies were from the same source and prepared in the same manner at Los Alamos. Titanium dioxide represents a particle of low toxicity, often used as a "negative control" for comparison to other materials. The titanium tetrachloride aerosol actually consisted of the hydrolysis products of the parent compound, and was thought most likely to be $TiCl(OH)_3$. The hydrolysis products collected in the lung and persisted as

TABLE 2
Pulmonary Carcinogenicity in Rats Exposed Chronically to Poorly-Soluble Particles

Material (Reference)	Exposure Rate ($mg \cdot hr \cdot m^{-3}/wk$)	Lung Tumor Incidence Fraction	(%)
Raw Oil Shale (Holland et al., 1986)	0	0/164	0
(Mauderly et al., 1986)	98[a]	0/110	0
(Holland et al., 1986)	2088[a]	15/59	25.4
Retorted Shale (Holland et al., 1986)	0	0/164	0
(Mauderly et al., 1986)	103[a]	4/109	3.7
(Holland et al., 1986)	2400[a]	11/59	18.6
Titanium Dioxide (Lee et al., 1985)	0	2/156	1.3
	165	3/146	2.1
	825[a]	1/149	0.7
	4140[a]	36/151	23.8
Titanium Tetrachloride Hydrolysis Products (Lee et al., 1986)	0	2/156	1.3
	3	0/152	0
	27	1/155	0.6
	267[a]	7/143	4.7
Petroleum Coke (Klonne et al., 1987)	0	0/96	0
	177[a]	2/87	2.3
	534[a]	12/92	13.0
Toner (Mermelstein et al., 1989)	0	3/100	3.0
	11	1/100	1.0
	45[a]	0/100	0
	163[a]	3/100	3.0
Kevlar Fibrils (Lee et al., 1988)	0	1/137	0.7
	2	1/133	0.8
	7	1/132	0.8
	13[a]	8/137	5.8
	47[a,b]	11/92	12.0

[a]Exposure levels for which dust overloading was suggested from the reported signs of particle retention, slowed clearance, inflammation, epithelial proliferation, fibrosis, or lung weight.
[b]Exposed for one year, followed by one year of observation.

solid particles. The petroleum coke was micronized raw "sponge" coke consisting of approximately 90% carbon and 2% benzene-extractable organic compounds by mass. The toner was 90% styrene/1-butylmethacrylate copolymer and 10% high purity carbon black. The Kevlar aramid fibrils were curled and branched, with a ribbon or tape-like configuration. The reported size distribution indicated that approximately 18% of the fibers were under 5 μm in length and approximately 22% were over 20 μm in length.

The tumor types observed in these studies included bronchoalveolar adenomas, bronchoalveolar adenocarcinomas, keratinizing squamous cysts, keratinizing cystic squamous cell carcinomas, and noncystic squamous cell carcinomas. The terminology for these tumors was inconsistent, and whether or not the keratinizing cystic lesions should be termed "tumors" at all remains in debate. All of these lesions have been included as "tumors" for the present comparison because that terminology is most prevalent and was used in all of the diesel exhaust studies listed in Table 1. The oil shale, titanium dioxide, titanium tetrachloride (hydrolysis products), and Kevlar fiber

exposures induced both adenomatous and squamous tumors. The petroleum coke exposures induced only keratinizing squamous cysts. Only adenomas were observed in control and exposed rats in the toner study. The tumors in all of these studies occurred late in the exposures, as did those in the diesel exhaust studies. The first tumor in the oil shale studies was reported to have been observed after 18 months of exposure, and most occurred after 21 months. Tumors resulting from Kevlar exposure were also reported to have occurred after 18 months. The times of tumor observation were not reported for the other studies; however, the reports suggest that the tumors were primarily observed at the final, 24-month sacrifice and, like those from diesel exhaust, had little effect on mortality.

The reports listed in Table 2 did not describe the statistical significances of the tumor responses; therefore, the reported fractions of rats having lung tumors are listed in the table. From these data, one can see that the tumor incidences were increased above control levels in all but the toner study, and were probably significantly increased in all others with the possible exception of the titanium tetrachloride study.

The data from Table 2, with the exception of those from the toner study, are plotted in Figure 2. The curve for diesel exhaust represents the function shown in Figure 1. The lines for the other materials were drawn by connecting points for individual exposure groups. Only the tumor responses of the exposed groups are shown; the control (zero exposure) points were omitted for clarity.

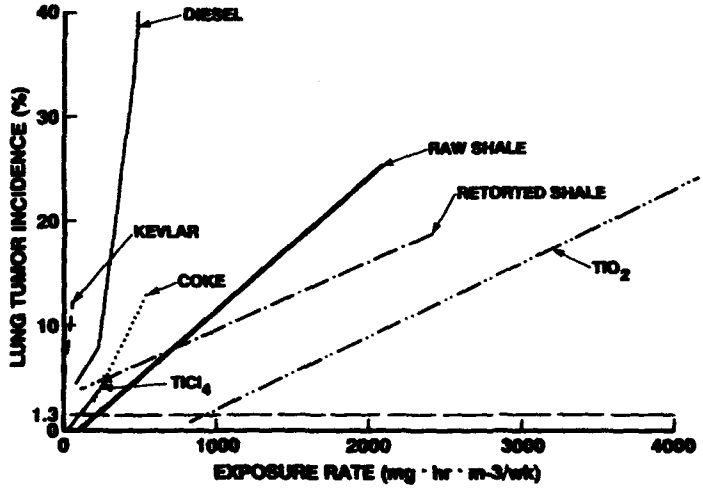

FIGURE 2. Exposure Rate-Tumor Response Relationships For Lung Tumors in Chronically-Exposed Rats. Data from Table 2 are plotted, excluding data for toner and the points for tumor incidences among controls. The dashed line represents the upper limit of the control lung tumor incidence (1.3%) in any study.

The exposure-response relationships shown in Figure 2 demonstrate that the results of chronic inhalation studies in rats provided a ranking of the relative carcinogenicities of these materials. The positions of the lower points (origins) of the curves give some indication of likely thresholds for detectable responses, and both the origins and the slopes give an indication of the relative carcinogenic potentials of the different materials. This comparison would suggest that the decreasing order of carcinogenic potential would be Kevlar fibers, diesel exhaust, petroleum coke and titanium tetrachloride (indistinguishable), oil shale, and titanium dioxide. Higher exposure rates for titanium tetrachloride would be required to distinguish its carcinogenic potential from that of petroleum coke. From the graph, one might conjecture that retorted oil shale has a lower threshold for response than raw

shale, but the strength of the data do not justify attempting this interpretation.

It is noteworthy that all of the exposure groups in Figure 2 that had significantly increased lung tumor incidences and essentially all groups suggesting an elevation of tumor incidence were judged from the information given in the reports to have been exposed under overloading conditions . This might suggest that the overloading itself was in some way responsible for tumor induction, and that the differences among the inhaled materials were simply due to differences in the rates of exposure which caused overloading. This hypothesis is negated by the fact that not all groups exposed under overloading conditions had increased lung tumor incidences. Toner was included in Table 2 because lung tumors did not appear to be induced by toner, even though the highest exposure level was clearly in an overload condition. For all other materials except perhaps Kevlar fibers, tumor incidences were not significantly increased in the intermediate exposure groups, although they appeared from the reported histopathology to have been overloaded with particles. These findings suggest that, although dust overloading might contribute to carcinogenicity, it does not necessarily cause carcinogenicity.

It is useful to consider that, if no groups had been exposed to these materials under overloading conditions, we would have little information on the relative carcinogenic potentials of the materials used in this comparison. By having tumor data from more than one exposure rate (level) per material, we have response curves that suggest the relative hazards from these materials. By recalling that the NTP Toxicology Design Review Committee implied that 100 mg/m^3 might be an upper bound for useful exposures (Lewis et al., 1989), one can calculate that this level would result in an exposure rate of 3000 $mg \cdot hr \cdot m^{-3}/wk$ if a standard NTP bioassay exposure schedule of 6 hr/day, 5 d/wk were used. Only the highest exposure level for titanium dioxide exceeded this rate in the present comparison (Table 2).

The points raised above are further supported by information from reports of chronic exposures of rats to other materials in studies which did not meet the criteria for inclusion in the preceding comparison. Studies of the carcinogenicity of quartz in long-term rat exposures (Dagle et al., 1986; Holland et al., 1986; Muhle et al., 1989) used single exposure levels of different types of quartz. The grouping of the data together in a single exposure-response curve is questionable due to the known differences in the toxicity of quartz and other crystalline materials caused by differences in surface structure (Wiessner et al., 1988). Wehner et al. (1986) exposed rats chronically to volcanic ash at two concentrations, but gave incomplete data on lung tumor incidences among the groups. Rats have been exposed chronically to coal dust (Martin et al., 1977; Lewis et al., 1986), but the studies included single exposure levels of coal from different sources and presumably having different compositions. Lung tumor induction has been reported in rats exposed chronically to antimony trioxide and antimony ore (Groth et al., 1986), but again, only single exposure levels were used.

Although the exposure rate-lung tumor response relationships in these studies cannot be examined in the same detail as those of the studies including multiple exposure levels, one can obtain a suggestion of the relative carcinogenicities of these materials from viewing the relationships for single exposure rates, as shown in Figure 3. Results of two of the quartz exposures (Holland et al., 1986; Muhle et al., 1989) suggest that quartz has high carcinogenic potential relative to the other materials, but the results of Dagle et al. (1986) show a much smaller response to quartz. The reason for the difference between responses in the Holland et al. and Dagle et al. studies is unclear, since both used Min-U-Sil quartz (Pennsylvania Glass and Sand Co.). The Muhle et al. study used DQ-12 quartz (Bergbauforschung, FRG), which is known to be a highly toxic form. Lung tumor incidences were not significantly increased by coal dust in the Lewis et al. (1986) study or by volcanic ash in the Wehner et al. (1986) study. The increases in lung tumor incidence caused by coal dust in the Martin et al. (1977) study, by quartz in the Dagle et al. (1986) study, and by antimony trioxide and antimony ore in the Groth et al. (1986) study all occurred at exposure rates of approximately 1000 $mg \cdot hr \cdot m^{-3}/wk$ or greater. This suggests that these materials have

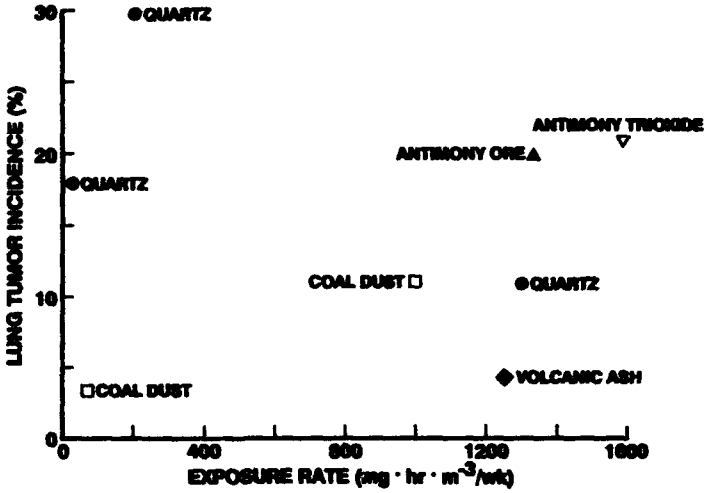

FIGURE 3. Exposure Rate-Tumor Response Relationship for Lung Tumors in Rats Exposed Chronically to Dusts. The figure illustrates carcinogenic responses to dusts for which data from multiple exposure levels are not available (see text for studies included).

relative carcinogenic potentials which approximate or exceed that of shale dust and which clearly exceed that of titanium dioxide.

Considerations in Using Relative Carcinogenicity Data from Overloaded Rats

The above exposure-response comparisons clearly yield a relative ranking of the carcinogenic potentials of the materials compared. This is precisely the purpose (hazard identification) of carcinogenicity bioassays: determining if a material can cause cancer and placing its relative carcinogenicity in some perspective. The troublesome fact remains, however, that this information has an unknown and, perhaps at best, a very limited value for predicting quantitative risks to humans exposed at lower concentrations and for different times. The most serious problem in extrapolating the low end of the exposure-response curves from animals to man is our lack of understanding of the mechanisms by which the lung tumors are induced in animals (or man), and whether or not the mechanisms operative in animals may be operative in man. This issue was recently summarized in regard to diesel exhaust (Mauderly et al., 1990), and is a topic of much current debate (Ames and Gold, 1990).

It is now broadly appreciated that cancer is a complex, multistep process. Although intense research continues to reveal many genetic and other cellular changes in precancerous and cancer cells, we do not yet know the complete series of minimum requisite steps for any tumor type. It has been speculated that exposures which cause prolonged increases in cell division rates, as lung overloading exposures do, may cause increased tumor expression by mitogenesis-induced amplification alone, without being the cause of any molecular change specifically required for conversion of normal cells to cancer cells (Ames and Gold, 1990). It is conceivable, for example, that there is no risk for cancer in humans from the materials compared above if humans are not exposed in regimens which cause significantly increased mitogenesis in man. Further, it is possible that the laboratory rat is uniquely sensitive to mitogenesis-induced cancer or amplification of carcinogenesis, and that this mechanism is not operative at all in man. These issues remain unresolved.

Despite the above caveats, animal bioassays remain the strongest tool available at this time for evaluating carcinogenic hazards. Proven human carcinogens are carcinogenic in animals. In its summary of information to

date in 1987, the International Agency for Research on Cancer (IARC, 1987) listed 50 agents as having sufficient data for classification as human carcinogens. Of those 50, there was also sufficient data for evidence of carcinogenicity in animals for 22 (44%), limited data for 7 (14%), sufficient evidence for lack of carcinogenicity in animals for none, and inadequate or no data from animals for 21 (42%). Importantly, although there were many materials shown to be carcinogenic in animals for which insufficient data exist for evaluation in man, there were no known human carcinogens proven to be noncarcinogenic in animals. The same relationships pertain if the review is limited to inhaled materials. Cigarette smoke is often cited as an exception, but it is not considered so by IARC. It is now widely recognized that the historical inhalation exposure methods and differences between man and rodents in deposition of smoke particles are likely reasons why inhaled cigarette smoke has not caused significant increases in lung cancer in rats, although a significant increase in total respiratory tract tumors has been demonstrated (Dalbey et al., 1980). In the case of diesel exhaust, although soot-induced pneumoconiosis of the magnitude observed in heavily exposed, overloaded rats has not been observed and is not expected in man, there is evidence that diesel exhaust is a weak carcinogen in man (Garshick et al., 1987; 1988) and there is also a tempting approximation of the presumed dose-response relationship in man to the lower end of the dose-response curve in rats (Mauderly et al., 1990). Thus, cancer bioassays have not yielded false negatives; the issue of concern is the likelihood of false positives.

Despite the uncertain direct relevance of data from overloaded rats to human carcinogenesis, there is interpretive value in knowing the threshold for overloading and the animal tumor response at that threshold. There are good reasons for using high exposures to establish exposure-response or dose-response curves; the difficulty lies in determining the upper limit of exposures providing useful information. It appears that extending the exposures up to the "maximum tolerated dose" as classically defined might not always be prudent (Ames and Gold, 1990). A useful alternative for poorly soluble, respirable particles might be to set the highest exposure level to achieve at least minimal overloading. For materials not expected to cause overloading in human exposures, this would ensure that the animal exposure range encompassed the full range of potential human responses. This approach also incorporates the value of results from overloaded animals for comparing relative carcinogenicities among materials.

RELEVANCE OF LUNG BURDENS OF PARTICLES IN OVERLOADED RATS TO POTENTIAL LUNG BURDENS IN HUMANS

Comparison of Accumulations of Inhaled Particles in Rats and Humans

The second criterion for the usefulness of data obtained from animals exposed under overloading conditions (see Purpose) was that either humans might be exposed under conditions like those used in the animal studies or that they might accumulate lung burdens of particles similar to those accumulated in the animal studies. There are exposures of humans to high concentrations of particles in occupational settings, although these represent quite limited populations. Although documentation is limited, there is anecdotal evidence that some miners are exposed to diesel soot in concentrations in the range of 1 mg/m^3, which is acceptable under the present coal mine dust standard of 2 mg/m^3 (OSHA, 1989). Workers in other environments can be chronically exposed to otherwise unregulated (nuisance) dusts at total concentrations up to 15 mg/m^3, and up to 5 mg/m^3 of respirable dust (OSHA, 1989). An occupational exposure to dust at 5 mg/m^3 for 8 hr/day, 5 d/wk would result in a weekly exposure rate of 200 $mg \cdot hr \cdot m^{-3}$/wk, a rate exceeded by only 6 rat exposure groups in Table 2. Thus, the potential exists for chronic human exposures within the range of those used in the rat bioassays.

The potential of humans for accumulating lung burdens of particles similar to those encountered in the rat studies was explored by modeling the lung burdens produced in man and rat by the same exposures. The modeling

approach was that described by Snipes et al. (1983) and Snipes (1989). It was first assumed that humans and rats were exposed for 8 hr/day, 5 d/wk, and that the pulmonary particle deposition fractions and clearance rates were normal. Deposition and clearance rates for humans were those derived from studies of dogs, which were shown by Snipes et al. and others to be a reasonable model for man. This gave the accumulation of particles in the lung if clearance remained normal. The accumulation was then modeled assuming that the deposition rate remained normal but that clearance was impaired to the same degree as observed in rats exposed chronically to diesel exhaust in the ITRI study (Wolff et al., 1987). The model had previously been shown to provide a good fit to the lung burdens observed in the exhaust-exposed rats (Snipes, 1989). It was found that, in order to fit the accumulation of lung burdens of soot in the rats, the short-term clearance component was required to be accelerated slightly and the long-term clearance component was slowed. Although different absolute rate constants were used for rats and man, clearance was assumed to be altered in the same proportion to the respective normal values of the two species. The projections were extended to 700 days to approximate the length of rat bioassays. All lung burdens were expressed in mg particles per g lung.

Figure 4 shows the particle lung burdens predicted for both normal and impaired clearance in rats and humans inhaling diesel exhaust or other materials having the same particle size characteristics at a concentration of 1 mg/m^3. It can be seen that, with normal clearance, rats and humans would be predicted to reach similar lung burdens at 700 days. Such an exposure may alter clearance in rats however, and if so, the 700 day lung burdens of rats would be approximately 3 times greater than those of humans with similarly altered clearance. Although the curve for altered clearance for humans remains below that for normal clearance during the 700-day period, it would be expected to eventually cross the curve for normal clearance, as it did for rats.

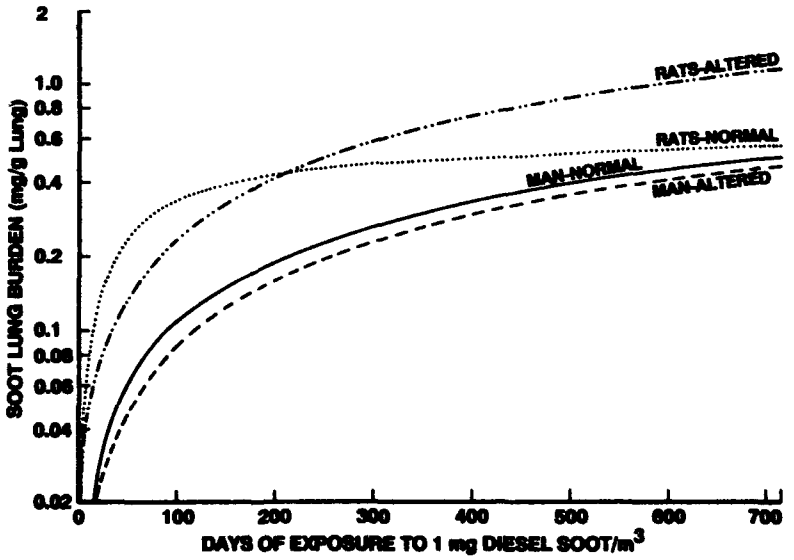

FIGURE 4. Accumulation of Lung Burdens of Diesel Soot in Similarly Exposed Rats and Humans. The curves represent lung accumulations of soot during exposures to 1 mg/m^3 for 8 hr/day, 5 d/wk in lungs with normal clearance and clearance altered as demonstrated in chronically exposed rats.

Figure 5 shows the accumulation of lung burdens in rats and humans exposed to a hypothetical nuisance dust 8 hr/day, 5 d/wk at a concentration of 5 mg/m^3. The dust particles are presumed to be polydisperse and to have a MMAD of 2 μm. Again, accumulations assuming both normal and impaired

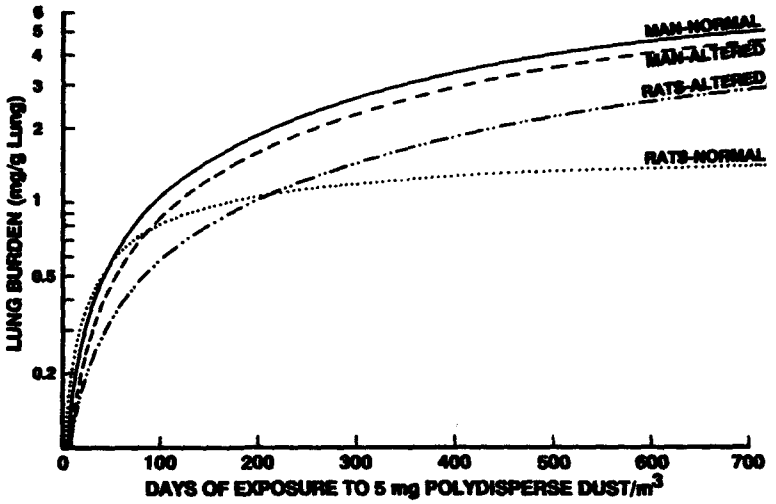

FIGURE 5. Accumulation of Lung Burdens of 2μm MMAD Dust Particles in Similarly exposed Rats and Humans. The curves represent lung accumulations of particles during exposures to 5 mg/m^3 for 8 hr/day, 5 d/wk in lungs with normal clearance and clearance altered as demonstrated in rats exposed chronically to diesel exhaust.

clearance are shown. The clearance impairment was the same as assumed for Figure 4. Due largely to the different deposition fractions for the larger particles, humans are predicted to accumulate larger lung burdens than rats, rather than smaller as for diesel soot. For the larger particles, humans are predicted to accumulate slightly over 1.5 times as much material by 700 days as rats.

The predictions shown in Figures 4 and 5 suggest that, for at least some particles, repeated occupational exposures of humans might result in the accumulation of lung burdens of particles similar to those observed in chronically exposed rats. Further, the lung burdens predicted for man fall above the approximately 2 mg/g lung which is generally agreed to be within the range associated with overloading (reduced clearance) in rats. These results suggest the possibility that dust overloading might indeed occur in some humans.

Comparison of Particle Accumulation in Rats and Humans During Similar Portions of Life Span

An attempt was made to model the potential accumulation of particulate lung burdens in humans over a working lifetime, because humans will be exposed longer than the 2 years common in rat bioassays. Any attempt to set corresponding ages of animals and humans yields questionable results; however, based on the comparison reported by Mauderly and Hahn (1982), a 20-year working lifetime for an adult miner (not an uncommon length) would be approximated by 11 months of exposure of adult rats. This interspecies age relationship was used to construct Figure 6, although the projection was carried beyond the 20-year (240-month) period for humans to encompass a 24-month exposure of rats. For this comparison, it was again assumed that the rats and humans were exposed 8 hr/day, 5 d/wk to diesel exhaust at 1 mg soot/m^3, as done for Figure 4. It was also assumed that clearance altered in both humans and rats.

Figure 6 shows that chronic exposures to diesel exhaust over major portions of the life spans of rats and humans may result in the accumulation of lung burdens of soot per g lung in humans that are greater than, or at least equal to, those in rats. It remains uncertain whether the lung tumor response during life-span exposures of rats approximates that which might occur during

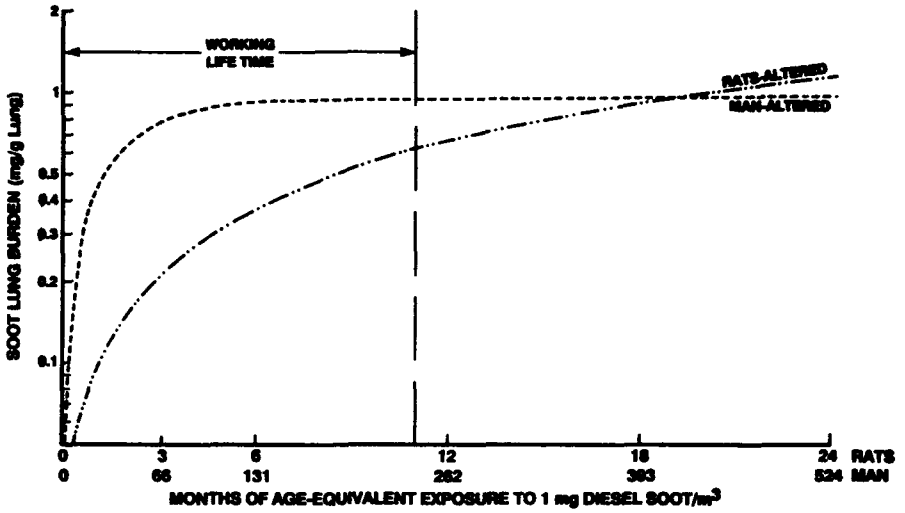

FIGURE 6. Accumulation of Lung Burdens of Diesel Soot in Rats and Humans Exposed During Equivalent Portions of Life Span. The curves represent lung accumulations of particles during exposures to 1 mg/m^3 for 8 hr/day, 5 d/wk in lungs with clearance altered as demonstrated in rats exposed chronically to diesel exhaust. Relative ages were estimated using the relationship reported by Mauderly and Hahn (1982).

life-span exposures of humans. The finding that lung tumors occur very late in the life span of chronically exposed rats in many studies raises the question about whether rats live long enough for carcinogenicity to be expressed as it might be in longer-lived humans. Although this question remains unresolved, the information shown in Figure 6 suggests that, over a comparably large portion of the life span, lung burdens in chronically exposed humans may exceed those in chronically exposed rats.

OTHER AGENTS OR CONDITIONS WHICH MIGHT CONTRIBUTE TO DUST OVERLOADING IN HUMANS

The third criterion for the relevance of information from rat studies including overloading exposures was that the phenomena associated with overloading in rats might occur in humans, if not from the exposure material of concern then from some other agent or disease. Because the carcinogenicity of several materials is expressed in rats only if "excess" particle retention, persistent inflammation, epithelial proliferation, or fibrosis occur, it appears that these abnormalities act to increase the expression of carcinogenicity. If it is true that these abnormalities amplify carcinogenesis, then it is not likely to be necessary that these abnormalities must result from the material of concern. Any condition increasing particle retention and concurrently causing the other abnormalities might increase the carcinogenic risk. Information on impairments of long-term particle clearance in man is very limited, and little is known about the potential contribution of impaired clearance to pulmonary cancer. Cigarette smoking is a common condition for which clearance impairments have been demonstrated in humans.

Accumulation of Lung Burdens in Smokers

Cigarette smokers represent an example population in which at least some of the features typical of overloaded rat lungs appear. Chronic smokers have been shown to have impaired clearance of particles deposited in the lung, as

recently reviewed by Oberdorster (1988), and also to have persistent inflammatory and tissue responses. The inflammatory and epithelial changes in smokers are centered primarily in conducting airways, rather than the alveolar lung, which might be related to the typical occurrence of airway, rather than parenchymal, tumors in smokers. However, the slowing of particle clearance from the deep lung would act to promote sequestration of larger lung burdens of inhaled materials than would accumulate in similarly exposed nonsmokers. It was recently reported (Mauderly et al., 1989) that rats exposed chronically to cigarette smoke had impairments of long-term (alveolar) clearance of tracer particles of a magnitude similar to clearance impairments reported for human smokers by Bohning et al. (1982). This lends support to the use of rats as models for humans with impaired clearance.

Figure 7 shows the accumulation of soot that might occur in lungs of smoking and nonsmoking humans exposed to diesel exhaust at 1 mg soot/m^3 for 20 years. For this comparison, the nonsmokers were assumed to have normal clearance and the smokers were assumed to have relatively normal short-term clearance, but a 1.8-fold delay in the long-term component of clearance, as reported for smokers by Bohning et al. The figure illustrates that steady-state soot lung burdens are predicted for both populations. It is expected that particle dissolution would eventually become a dominant factor in setting the steady-state lung burden of chronically exposed humans. The figure also illustrates that the lung burdens of smokers would exceed that of nonsmokers at all times, in contrast to the previous figures in which altered clearance resulted in initially lower lung burdens. This is because no speeding of short-term clearance was found in human smokers by Bohning et al., as it was for diesel exhaust-exposed rats by Snipes. The results indicate that the lung burdens of chronically-exposed smokers might reach steady states exceeding 1 mg soot/g lung. Although this comparison represents only one of several potential conditions which might impair clearance in human lungs or cause the other abnormalities which are associated with dust overloading in rats, it serves to suggest that rat inhalation bioassays including overloading exposures have relevance for potential human responses.

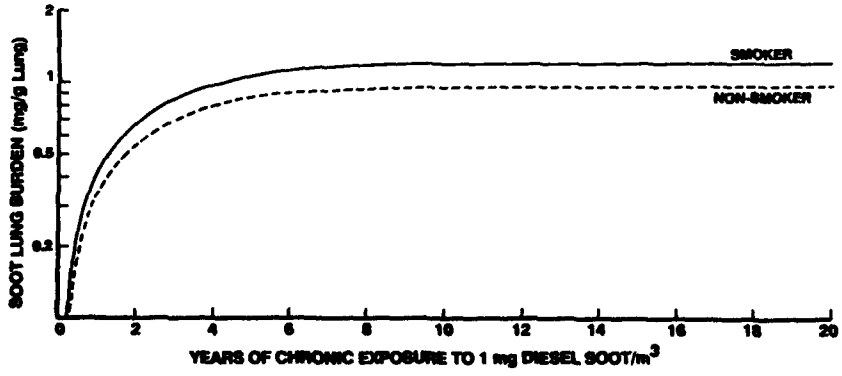

FIGURE 7. Accumulation of Lung Burdens of Diesel Soot in Human Smokers and Nonsmokers. The curves represent lung accumulations of soot during exposures to 1 mg/m^3 for 8 hr/day, 5 d/wk in lungs of humans with normal clearance and in lungs of humans with clearance altered as reported for human cigarette smokers by Bohning et al. (1982).

CONCLUSIONS

The potential usefulness of carcinogenicity data from studies of rats which include exposures causing particle overloading has been reviewed. It was shown that such studies can produce exposure-response information indicating the carcinogenicity of the test material relative to those of other materials. It was also shown that exposures resulting in particle overloading in rats might possibly result in similar lung burdens of particles in humans, and that some

occupational human exposures might fall in the range of animal exposures causing overloading. Finally, it was shown that lung accumulations of particles during chronic exposures of humans with clearance impairments might possibly approximate those in some of the rat studies in which carcinogenicity was demonstrated.

To ensure accuracy in using bioassay data for either hazard identification or dose-response extrapolation requires knowledge that the mechanisms by which the effect occurs in animals can operate in man, and our present knowledge is incomplete. We have information indicating that animal bioassays are useful for detecting human carcinogens, but have difficulty determining an appropriate upper limit for exposures in order to avoid false positives. It now appears that setting the upper exposure limit at the maximum tolerated dose as defined in the past might not always be appropriate. We do not yet have sufficient understanding to conclusively accept or reject bioassay data acquired under overloading conditions that fall within the range of the classically-defined maximum tolerated dose. Based on the premises and information in this review, it appears prudent for the present to continue inhalation carcinogenesis bioassays and to construct studies of poorly-soluble, respirable particles such that the highest exposure level results in at least minimal overloading. For these materials, and in the absence of predicted overloading in exposed humans, the "minimum overloading exposure" might be an acceptable substitute for the "maximum tolerated dose" in study design.

INFORMATION NEEDS

There are several areas in which additional research needs to be conducted to provide information which will improve our ability to design and interpret inhalation studies in animals, extrapolate findings in animals to man, and better understand factors contributing to health risks from inhaled, poorly-soluble particles. To varying degrees, research is currently under way in most of these areas.

We need to better understand the relative roles of particle-borne organic carcinogens and the carrier particle in the induction of cancer and other effects from chronically inhaled particles. Several short-term studies have been conducted to examine the release of particle-borne compounds in the lung and their subsequent tissue interactions, metabolism and excretion. Long-term studies comparing the effects of chronically inhaled diesel exhaust to those of carbon particles are under way, and should serve to clarify the issue of the specificity of the rat's response to diesel exhaust. Chronic studies of other organic compounds inhaled as pure particles or on carrier particles are few, and this remains an area of need.

We need to better understand the kinetics of the movement of particles deposited in the alveolar region. Of particular interest is the rate of incorporation of particles into aggregated macrophages thought to represent a sequestration compartment. The movement of particles into interstitial and lymphatic locations is also of interest. The location of particles "sequestered" in the lung should have relevance to their potential for causing adverse responses.

There is continuing speculation about the potential role of particles sequestered in macrophages or in other locations in serving as a source of slow release of particle-borne chemicals, and the role this release might play in genotoxicity. It is not clear at this time what portion of the genotoxic compounds or metabolites binding to DNA might come from recently-deposited material and what portion might come from sequestered material.

There is a need for studies of the effects of co-exposure to other agents or concurrent lung disease on the expression of toxicity of inhaled materials. It is not known how concurrent exposure to organic or inorganic dusts might influence the expression of toxicity of materials inhaled at levels that might not exert detectable toxicity alone. The promotion of lung carcinogenicity by the inflammatory and proliferative responses to a second chronically inhaled material is unknown. Of particular note is the absence of studies examining the influence of chronic cigarette smoking on the expression of toxicity from other chronically inhaled materials. Similar studies could be done using other models

of lung disease incorporating persistent inflammatory and proliferative responses.

Finally, and perhaps most importantly, there is a need for a better understanding of the fundamental mechanisms of carcinogenesis. Of particular concern is the identification of the requisite cellular and molecular steps among the rapidly-expanding assortment of changes that are being identified as associated with carcinogenesis. Ultimately, the ability to choose wisely from among our toxicological tools and to use those tools to best advantage in assessing risk to humans will be determined by the quality of our understanding of the mechanisms by which different cancers are induced by different materials.

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Lung Overload: A Challenge for Toxicology

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ABSTRACT

Chronic lung overload may result in the development of fibrosis and of tumors in the lung parenchyma. The essential question that must be answered is whether there exists a threshold of exposure below which these effects are unlikely to occur. Both threshold and non-threshold mechanisms appear to exist for the two conditions, as illustrated by selected examples. Definition of a threshold is often driven by present analytical approaches. Mechanistic studies should not only address development of lesions, but also examine events that determine tissue recovery.

STATEMENT OF PROBLEM

The collection of observations that has been called "the condition of lung overload" has become an issue of considerable scientific and also of practical interest, as evidenced by the present meeting. The problem may be stated briefly as follows: in inhalation studies, is it really legitimate to expose animals to concentrations of an inhalant that will "overload" the lungs, to the extent that many defense mechanisms break down or become inoperable? And how do we interpret our observations should signs of toxicity be observed under conditions of overload? Have we created a meaningless disease, not important for man, or should we take the findings as a warning sign?

The problem is, of course, not unique to inhalation toxicology. In practically all chronic toxicity studies, at least one group of animals is exposed to what has been called the "Maximum tolerated dose" (MTD). It can be argued that a MTD really constitutes a form of systemic overload. In animals fed the MTD of a given compound, alternative metabolic pathways may become opened up, some of them

Key Words: Fibrosis, Collagen, Lung, Cancer, Particles, Overload, Threshold

unphysiological. Natural detoxification mechanisms that often are induced and functional may, under overload conditions, become overwhelmed and inoperational. Continued use of the MTD in carcinogenesis bioassays is at least as often debated and questioned as are exposure protocols that involve apparently unduly high concentrations of inhaled particles. Furthermore, it is common-day experience that tells us that outright overload of a system may have dire consequences, whereas exercise of some care in not overstepping boundaries may allow us to carry on without any untoward health effects: alcoholic cirrhosis of the liver is most likely to occur only after prolonged, excessive daily intake of ethanol. Moderate consumption does not appear to have such dramatic untoward health effects. Ultimately discussions and conclusions will depend heavily upon one's assumption or belief whether or not an observed effect shows a threshold level for its expression.

The use of what appears to be excessively high doses has some rationale. The results of toxicity studies in which comparatively small populations of animals are exposed to high doses of a chemical really serve as a surrogate for the human situation, where large populations are exposed to much smaller amounts of the chemical. Once this premise is accepted, it must be remembered that toxicity testing really should address two fundamental questions. One question is whether a given compound has the potential to cause a certain toxicity. The other question is whether this potential is likely to be realized under conditions approximating or perhaps even equal to actual or anticipated human exposure. This old problem is perhaps better understood in some more modern terms: hazard identification and risk assessment. The two questions do not address the same problem and it might actually be quite improper to seek answers to the two problems with only one experimental design or protocol.

Why then are we not content to simply establish the potential of any given agent to do harm in one set of experiments and then, in a second series of studies, to examine whether this harm would be expressed under "realistic" exposure conditions, relevant for man? The answer is straightforward: certain "potentials" are simply not acceptable; carcinogenic potential being the one of most concern. For reasons too numerous to be listed here, some years ago it became socially and politically unacceptable to expose people to any agent having "carcinogenic potential". This development reached its zenith in the famous Delaney amendment. At present, it is realized that to attain zero exposure is impossible; therefore, efforts are made to reduce exposure to an absolute minimum. As a consequence, it is politically unacceptable to expose people to any carcinogenic agent if it can be avoided at all - even when there are orders of magnitude between exposure conditions that reveal "carcinogenic potential" and exposure conditions that might be encountered in real life. On the other hand, many other potential toxic effects that are not feared to the same extent as carcinogenesis are more readily accepted and acceptable.

Overload of the lung with so-called inert particles can produce several functional and morphological lesions. The two most prominent toxic effects, found in a number of animal studies, appear to be accumulation of abnormal amounts of hydroxyproline (attributed to collagen) in the lung and development of tumors. It can thus be said that inert dusts may have fibrogenic and carcinogenic potential. We need to acquire some understanding about potential mechanisms underlying the development of the two forms of lung lesions to properly evaluate these issues.

PULMONARY FIBROSIS

It now appears that pulmonary macrophages may play an important role in the development of pulmonary fibrosis, particularly fibrosis

brought about by inhalation of so-called nuisance dusts. Alveolar macrophages can act as effector cells and release multiple factors which eventually stimulate or inhibit the production of collagen by lung fibroblasts. Several in vitro and in vivo studies are available to document this important interaction between macrophages and lung fibroblasts (Reiser and Last, 1986).

While all these ongoing and exciting studies advance our understanding of fibrogenesis, they almost exclusively address one problem *only*: what are the conditions that trigger an increased production of collagen in the lung? There are very few, if any, concomitant studies that would address the equally important question: what are the mechanisms that determine whether once synthesized and secreted by fibroblasts, collagen stays in the lung, may become chemically modified or may even disappear? The question is not a trivial one. Two examples of pulmonary fibrosis should illustrate this point. In both experiments, the fate of pulmonary collagen was followed up to one year after a single exposure to a compound having fibrogenic potential.

In one experiment, mice received a single intraperitoneal injection of a lung-specific toxicant, butylated hydroxytoluene (BHT). BHT is known to produce acute type I alveolar cell and capillary endothelial cell necrosis, followed, within 2 to 4 days, by extensive type II alveolar cell proliferation and essential repair of the blood-air barrier. The acute phase of the lesions looks rather dramatic and conveys the picture of a diffusely damaged lung; however, after about 2 to 3 weeks, the lungs look essentially normal again. Nevertheless, acute lung injury produced by BHT results in some accumulation of pulmonary collagen, as evaluated biochemically as total lung hydroxyproline content. Two to three weeks after administration of BHT, total lung collagen content is about 40% higher than in control lungs. There also is an increase in the relative amounts of type I collagen. The lung hydroxyproline content is observed at this elevated level for about 6 months; however, one year after the original insult, it is only about 20% higher than in age-matched controls and the ratio of type III to type I collagen has returned to normal. Morphologically, the early lung lesions are for all practical purposes resolved; there is certainly no longer any evidence of pulmonary fibrosis (Haschek et al., 1982).

The second example involves cyclophosphamide. Again a single intraperitoneal injection of a drug causes acute lung damage. Initial lesions after administration of 100 mg/kg are minor and 3 weeks later there are in the lung parenchyma a few scattered areas displaying slight interstitial infiltrates and some foci of foamy alveolar macrophages. At this time, total lung hydroxyproline is about 30% higher than in controls. However, contrary to what was seen with BHT, the lesions produced by cyclophosphamide do not resolve or disappear, but become gradually worse. The number and size of interstitial infiltrates increase and, one year after administration of a single dose of cyclophosphamide, total lung hydroxyproline is 55% higher than in the lungs of control animals (Morse et al., 1985). It may be notworthy that the observations made about the chronic progressive lesion of cyclophosphamide in this initial study were recently and independently fully confirmed (Travis et al., 1990).

Two drugs, each one given by the same protocol, a single administration, thus initially produce lesions that appear to be similar. The exact mechanism by which either agent causes lung damage is not known with certainty; all that is known is that both agents presumably require metabolic activation to more reactive intermediates for their fibrogenic potential to be expressed. However, the long-term development of the drug-induced lesion is dramatically different for the two agents: after BHT, injury seems to eventually resolve, whereas after cyclophosphamide there is a progressively worsening disease. Interestingly, similar observations

have also been made in two long-term studies following a one-time exposure to a toxic metal aerosol: one year after exposure to an aerosol of CdCl2, the morphologic lesions that developed initially were mainly resolved (Martin and Witschi, 1985). After inhalation of BeSO4, on the other hand, lesions appear to be progressive over time (Sendelbach et al., 1989).

These examples illustrate a need for research in a field that, for all practical purposes, is often overlooked in experimental toxicology. Very few studies have been done to examine underlying mechanisms at the cell and tissue level that might determine whether initial lesions resolve with time or become progressively worse. For example, there is evidence suggesting that during the development of acute fibrotic lesions there are more cells synthesizing collagen in the damaged lung (Kehrer and Witschi, 1980). However, in slowly progressing lesions, it seems that now the number of cells remains unaltered; the cells appear to synthesize collagen at a higher rate than normal (Witschi et al., 1985; Tryka et al., 1986). There is also a paucity of information on mechanisms underlying the development of chronic degenerative lesions following toxic injury. An additional consideration relates to dose rate. In the BHT-pulmonary fibrosis model mentioned before, a single high dose of BHT produced a definite fibrotic response. The same cumulative dose of BHT, given in several smaller doses administered over a prolonged period failed to produce any abnormal accumulation of pulmonary collagen (Haschek et al., 1982). The role of dose rate in the pathogenesis of chronic tissue lesions needs to be better understood, as does the potential role of toxic interactions. In summary, as far as non-carcinogenic lesions are concerned, a challenge to toxicology will be not only to understand mechanisms of acute injury, but also to understand mechanisms of tissue recovery, progression of lesions and points of no return at which lesions become irreversible.

LUNG TUMORIGENESIS

Particle overload of the lung also has been associated with the development of lung cancer. That lung cancer should develop in animals exposed to Diesel exhaust emissions is comparatively easy to understand, whether particle overload occurs or not (Mauderly et al., 1989). After all, Diesel exhaust particles are known to contain many carcinogens, some of them being quite potent, and it should not be a surprise that exposure to agents known to have mutagenic potential in multiple in vitro tests should produce lung tumors in vivo. However, development of lung cancer after inhalation of so-called "nuisance dusts" is somewhat more difficult to understand (Lee et al., 1985). There are two problems: one is, why are certain dusts called "nuisance dusts"? Most probably because they are comparatively inert, i.e. devoid of apparent toxicity, in short term, high exposure studies. Also they do not appear to have genotoxic potential in conventional in vitro assays for genotoxicity. Nuisance dusts do not seem capable of interacting directly with DNA and thus are not believed or assumed to be truly and directly carcinogenic. The second problem is that nuisance dusts seem to be capable of triggering cell proliferation in the lung.

Particles and the biological responses they elicit may well be of more importance in the development of lung cancer than is generally assumed. It might be worth remembering that the first truly successful and reproducible method of inducing bronchogenic carcinoma in experimental animals deliberately took advantage of pulmonary overload. Saffiotti et al. (1968) produced the first reliable model of lung cancer in experimental animals by instilling the carcinogen benzo(a)pyrene into the lungs of hamsters, together with a carrier dust of ferric oxide. Ferric oxide particles were thought to be

crucial in inducing the desired result. They not only would help to carry the adsorbed carcinogen, benzo(a)pyrene, into the deep lung, but they would ascertain that, once deposited, the benzo(a)pyrene adsorbed to the insoluble particles would remain for some length of time at the site where it would be possible to produce the desired result. Furthermore, the particles themselves were also believed to induce secondary changes in the lung tissue, such as inflammatory events and cell hyperplasia, conditions thought to be favorable for the ensuing development of neoplastic lesions.

It now appears to be well established that cell hyperplasia within the respiratory tract may play an important role in the pathogenesis of many tumors. It is well known that human lung cancers often develop within or in the vicinity of scarred lung tissue (Churg, 1988). In several animal models of lung cancer it has been shown that a stimulus capable of producing cell hyperplasia within the respiratory tract often enhances development of tumors (Little et al., 1978; Keenan et al., 1989 a, 1989 b). Of particular interest is the observation that in many experimental situations cell hyperplasia can be caused by non-carcinogenic stimuli. Such an event then greatly enhances the efficiency of an accompanying treatment with a carcinogen. For example, concomitant exposure of rats to the carcinogen benzo(a)pyrene and to the air pollutant SO2 has been said to enhance respiratory tract carcinogenesis (Laskin et al., 1976). Another common air pollutant, ozone, may under certain conditions enhance development of chemically induced lung tumors in strain A mice (Hassett et al., 1985). In animals exposed to ozone alone, an increased number of lung tumors can be observed (Hasset et al., 1985; Last et al., 1987). This finding might implicate ozone as a complete carcinogen; however, it is also possible that increased cell turnover caused by ozone inhalation simply increases the number of cells in the strain A mouse lung at risk of undergoing "spontaneous" transformation. Evidence has also been provided to document that increased cell turnover brought about by such stimuli as tracheal wounding or instillation of 0.9% solution of NaCl may be enough to enhance and to modify carcinogenicity in hamster lung brought about by co-administration of chemical or physical carcinogens (Keenan et al., 1989 a, b).

It is therefore not surprising that excessive accumulation of particles in the lung can be associated with tumor development, particularly not in such cases where the particles have been found to carry highly carcinogenic materials attached to them. Tumor development may be somewhat more difficult to explain when particles are, by all current standards, "inert", e.g. do not have any evidence for mutagenic potential in the conventional in vitro assays. Is a mitogenic stimulus for lung cells then enough to activate the process of "endogenous carcinogenesis" (Loeb, 1989) or to act as a promoting stimulus? If this is the case, we will need better information on the process of tumor-enhancing or tumor-promoting stimuli than we have so far.

It was mentionned before that for carcinogenesis we do not admit to the existence of thresholds. On the other hand, for promoters we generally do. This is probably an artificial differentiation and it is certainly not based on experimental facts. In any conventional animal bioassays, a "threshold" or no-effect dose can usually be found. Bioassays, as presently conducted, contain several dose groups and tumor response in experimental animals is often identical to controls at the lower levels of exposure. The notion that carcinogens do not have a threshold does not come from animal experimentation but goes back to two facts: mechanisms of carcinogenicity are believed to involve alterations of the cellular genome. The theory became widely accepted at a time when very little if anything was known about DNA repair mechanisms and has since then been buttressed by an impressive amount of data obtained with in

vitro systems. The other reason is that the most exhaustive information on human cancer, the data obtained in radiation studies, is compatible with a conservative, i.e. linear, no-threshold model (Upton, 1989). It is of course also possible in every study with promoting agents to find a threshold for the so-called promoting agent. Since tumor promotion does not appear to involve direct and immutable interaction with the genome and since there are certainly no human data on the effects of promoters, it is acceptable to claim the existence of thresholds for promoters.

We now have a problem: how to be sure whether events known to be non-genotoxic, but also known to be involved in carcinogenesis will have threshold characteristics? Or to be more specific: is there a threshold for carcinogenesis by nuisance dusts? It has been already mentionned that there certainly is evidence for of spontaneous or endogenous production of initiated cells, waiting to be stimulated by promoting events (Loeb, 1989). Cell proliferation could be such an event. It is also an old notion in carcinogenesis that a round of cell replication is needed in an initiated cell, in order to "lock in" the changes in the genome. Are we to assume that events which cause cell proliferation in the lung will not do so at all below a certain dose - or will it simply be impossible with present methods to detect "subthreshold" effects? And if it is only a question of detection - how can we be sure that, below a certain dose, this particular mechanism of carcinogenesis still might work?

DISCUSSION

Two of the most salient features observed under conditions of lung overload are fibrogenesis and carcinogenesis. For both biological responses there are large gaps of knowledge about events occurring subsequent to initial "injury" of cells that determine whether subsequent events are reversible or irreversible. Analysis of the initial event only is not enough to predict future development of a toxic injury. Mechanistic studies on tissue recovery are an area of great future research needs and one in which the necessary tools and procedures may not yet have been demonstrated.

In the extreme, however, we can say that the concept of lung overload implies one of three scenarios to be understood. The first one is that there are thresholds to effects on the lung, and that overloaded lungs are unphysiological because doses administered exceed such thresholds whereas reduced exposure levels do not. The problem lies of course in the definition of a threshold and also in the definition what eventually will constitute an adverse effect. Present mechanistic research places much emphasis on the pathogenesis of the initial lesions, whereas the healing role of counterforces and of physiological damage control is often not appreciated enough.

The second possibility is that the mechanisms by which the lung responds to dusts vary with dose administered. If this is the case, all extrapolation from high to low doses of instilled or inspired dusts would become meaningless. And the third possibility is that our current assays of effects of dusts on the lung are comparatively insensitive and the signal to noise ratios are such that the current concept of overload simply reflects inadequacies of the methodology available to the lung toxicologist.

Lung overload has given many challenges to toxicology. The two biggest ones appear to be to learn more about mechanisms of tissue recovery and reversibility of lesions and to deal with the issue of thresholds. The first issue will need some more research. So does the second, but it also might need some rethinking of old premises and assumptions.

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Particle Overload in the Lung: Approaches to Improving Our Knowledge

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ABSTRACT

Lung overload is a condition characterized by (1) an overwhelming of normal clearance processes under certain exposure conditions, (2) resulting in lung burdens greater than predicted from disposition kinetics observed at low exposure concentrations, (3) with associated pathophysiological changes including altered macrophage function, inflammation and pulmonary fibrosis and (4) an uncertain association with an increased incidence of lung tumors in studies conducted in rats.

Our present knowledge is not sufficient to distinguish between the role of compound specific mechanisms and non-compound specific mechanisms in the development of the lung overload condition. This is of particular concern when assessing the potential human health risks of exposure to particles using information from inhalation studies conducted in rats. An improved knowledge base on this issue can be developed through appropriately designed and interpreted studies.

This paper (a) reviews the role of studies with an exposure-dose-response orientation conducted at multiple levels of biological organization in understanding and assessing human health risks for airborne particles, (b) discusses the overload condition with particular reference to understanding compound specific versus non-specific effects of inhaled materials, and (c) recommends approaches to the conduct and interpretation of inhalation studies with particulate materials conducted in rats.

INTRODUCTION

During the past decade a lung overload condition has been observed in a number of lifespan inhalation toxicity studies conducted with particulate materials in rats. This overload condition is characterized by an overwhelming of normal clearance processes under certain exposure conditions, resulting in lung burdens greater than predicted from disposition kinetics observed at low exposure concentrations. Associated pathophysiological changes include altered macrophage function, inflammation, and pulmonary fibrosis, and a possible association with an increased incidence of lung tumors. An

KEY WORDS: overload, exposure-dose-response, cytokines, lung cancer, lung fibrosis, lung burden, particles, fibers, rats

understanding of this overload condition in rats, in particular its possible role in the development of lung cancer, is crucial to the appropriate interpretation of studies conducted with inhaled particles in rats and the extrapolation of the results for assessing human health risks from exposure to these materials.

In this summary paper, I will (1) review the role of studies with an exposure-dose-response orientation conducted at multiple levels of biological organization in order to understand and assess human health risks for airborne particles, (2) discuss the overload condition with particular reference to understanding compound specific versus non-specific effects of inhaled materials, and (3) recommend approaches to the conduct and interpretation of inhalation studies with particulate materials conducted in rats.

Exposure-Dose-Response Orientation

In the context of toxicology and risk assessment, it is crucial that research on the pathogenesis of toxicant-induced disease should have a strong exposure-dose-response orientation (Figure 1). Our ultimate interest is not one of merely ascertaining if exposure to a toxicant causes disease, but what is the likelihood that the disease will occur at levels of exposure in the range to which people might be exposed. Steps can then be taken to minimize the potential for exposure at levels that would pose an unacceptable risk to people.

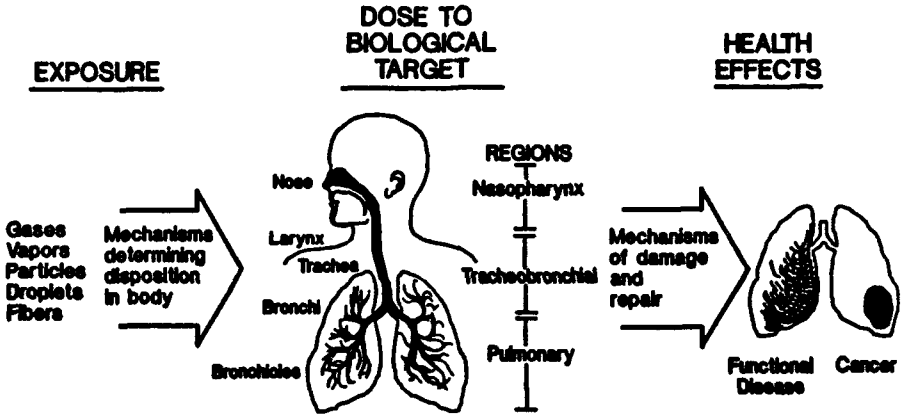

FIGURE 1. Interrelations in assessing health effects of airborne toxicants

The use of the term, dose, in the exposure-dose-response paradigm is essential to understand fully exposure-response relationships. This is especially the case recognizing that significant species differences can exist in the mechanisms that influence both exposure-dose and dose-response relationships. Further, the use of a dose term provides a means for integrating information from studies using sub-animal systems such as tissues or cells in which attention is focused on mechanisms interposed between dose and response.

Role of Multiple Biological Systems

Our ultimate goal is to understand the disease processes induced by toxicants, and through this understanding minimize toxicant-induced disease. If significant human exposure to the toxicant in question has occurred, it may be possible by studying exposed people to gain considerable understanding of the relationship between exposure and disease and, indeed, even gain insight into the mechanisms by which the disease is induced by the toxicant. Fortunately, human exposures of this significance have occurred for relatively few toxicants and, thus, with the exception of these few compounds data from people are not available nor is it likely to be developed.

In most cases, even for toxicants for which significant human exposures have occurred, it is likely that added insight into exposure-dose-response relationships for the material can be gained from studies with non-human systems. The use of multiple systems to study respiratory diseases induced by inhaled materials is illustrated schematically in Figure 2. With the opportunity available to study exposed people, the emphasis is on comparison of information gained in different systems.

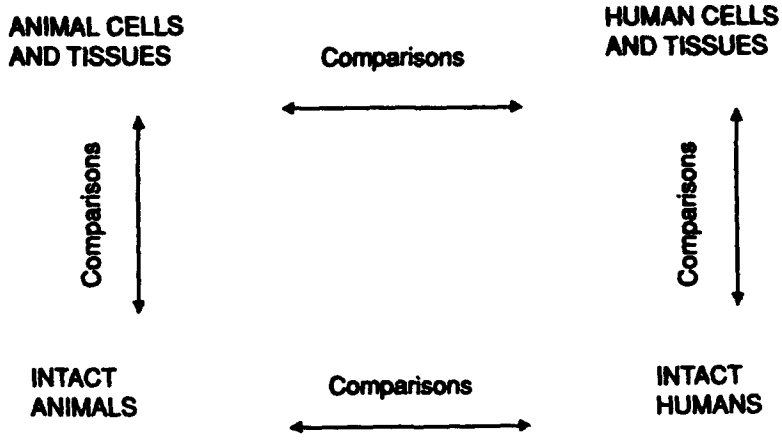

FIGURE 2. Use of multiple systems to study the pathogenesis of respiratory disease.

Without the opportunity to make observations in intact people, the experimental strategy depicted in Figure 2 must be altered as shown in Figure 3. The emphasis thus shifts to integrating information gained in studies with human cells and tissues and intact animals and making extrapolations to people. It is

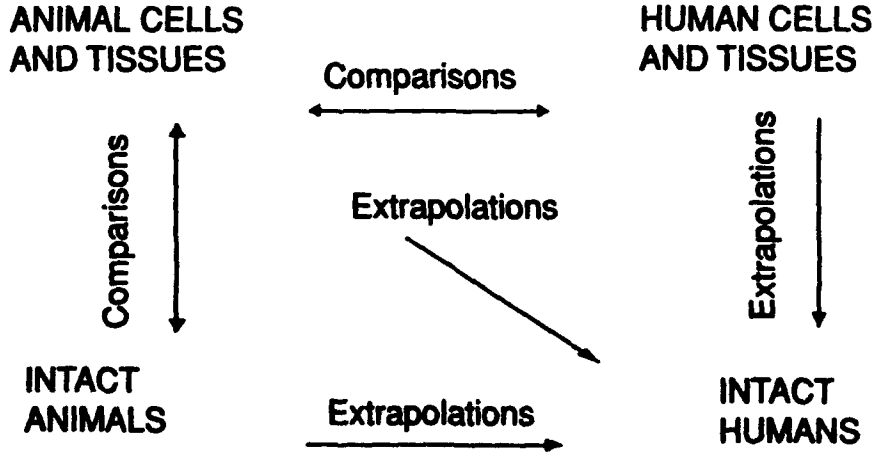

FIGURE 3. Use of multiple systems to provide a knowledge base for estimating human health effects.

essential to have available an approach that is not dependent upon data from exposed people since one of our goals is to be able to evaluate the potential for newly developed materials to cause disease in people. This must be done prior to introduction of the material into commerce and the associated potential exposure

of people to the material. This prospective orientation then provides the toxicological information base for determining how best to manage potential risks of the new material, i.e. at what level to set exposure limits for occupational, environmental or consumer exposure, what limits should be placed on use of the material, etc.

To a large extent, our major concern for the potential for induction of disease in people focuses on diseases like cancer or chronic respiratory disease that may arise after long-term exposure. This concern places a premium on being able to establish linkages between responses observed at early time periods following brief exposures and the ultimate manifestation of chronic disease with long-term exposure. This experimental strategy is depicted schematically in Figure 4. The left side of this figure indicate the collective studies that are required to provide sufficient knowledge of the pathogenesis of toxicant-induced disease in laboratory animals so that realistic projections can be made for the potential occurrence of disease in man. In my view, the appropriate studies that are likely to be conducted in the future will include lifespan bioassays to determine both the kind of diseases potentially induced by the material and the quantitative relationship of these diseases to exposure.

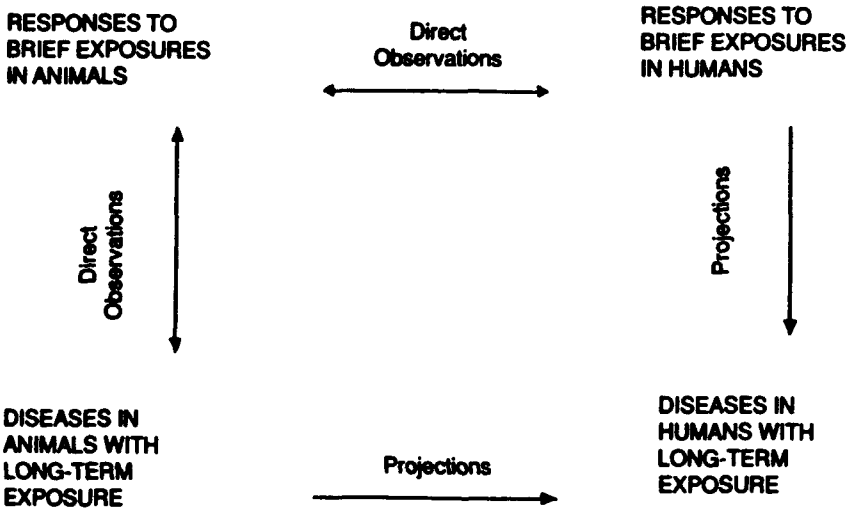

FIGURE 4. Interrelations between studies conducted in laboratory animals and human subjects.

To maximize the utility of bioassays for hazard identification, I believe it will become increasingly important to modify the way in which bioassays are conducted. Keeping in mind the need for an exposure-dose-response orientation, as much attention should be given to the influence of exposure on dose as traditionally has been given to the exposure-response relationship. In addition, the opportunity should be taken to obtain specimens, serially through the course of the bioassay and at the conclusion of the study, that can be used to give insight into the pathogenesis of diseases that may be induced. For example, a range of techniques are already available, and more will undoubtedly be developed, that give insight into changes in cell proliferation and the status of the genome of target cells.

Beyond the use of lifespan bioassays, I believe it will prove increasingly useful to conduct additional long-term pathogenesis studies when investigating the mechanisms of action for materials that have already demonstrated disease producing potential at some level of exposure in classical bioassays. The hallmark of such these long term pathogenesis studies should be severalfold. First, multiple exposure levels should be used that range from those having produced disease in the bioassay down to levels in the range of those that are likely to be experienced by people. Second, they should be of sufficient

duration so that the disease of interest, for example, cancer, will be observed. Third, the study or companion studies should be conducted in a manner that gives insight into the disposition with the body, at the level of target cells and molecules, of the inhaled material or its metabolites. And fourth, appropriate number of animals and experimental techniques should be used to give insight into the pathogenesis of the disease of interest.

Two major interrelated questions will be addressed by pathogenesis studies of this type. First, are the mechanisms by which the disease of interest is produced at high levels of exposure likely to be operative at lower levels of exposure such as those likely to be encountered by people i.e. to what extent is the disease exposure-level specific? And second, are the mechanisms observed in the rodent likely to be operative in exposed people, i.e. to what extent is the disease species-specific?

Exposure-Response Extrapolations

As previously noted, a key consideration in the use of information from studies conducted with laboratory animals is the confidence with which data obtained at exposure levels higher than those likely to be encountered by people can be used for estimating human health risks. This dilemma is illustrated schematically in Figure 5. Note, this is an issue not just with the use of data from studies with laboratory animals but also in using data obtained from *in vitro* studies or studies in which materials are injected into animals.

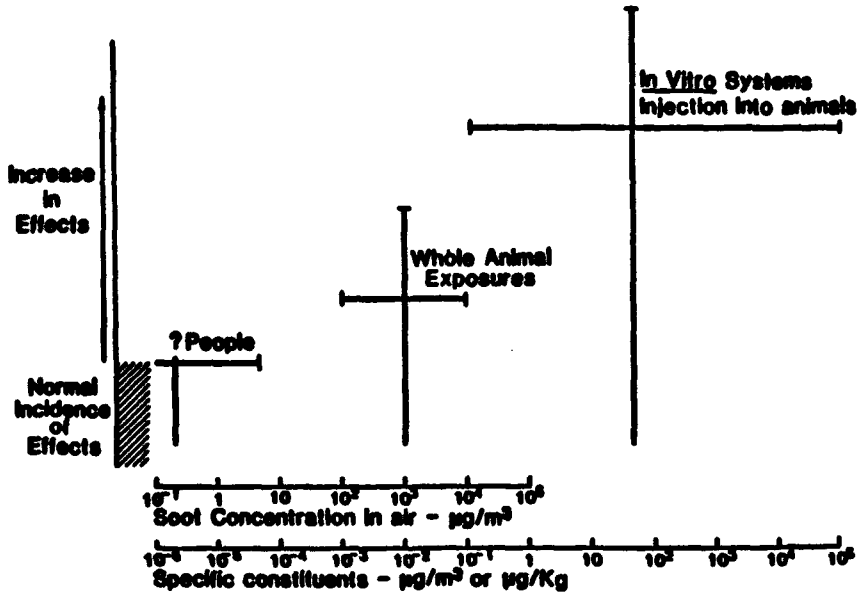

FIGURE 5. Exposure-response relations (The dilemma of extrapolation).

To elaborate on Figure 5, first, consider the vertical axis which is labeled "Increase in Effects." When considering effects, it should be kept in mind that the baseline is rarely zero. Typically, the effect of concern such as cancer or precursor changes occurs at some background level in the population being studied whether it be people, laboratory animals or cells. For each of the particular systems, a vertical bar is used to illustrate the range of effects that may be observed. A point to be emphasized is that the effects of ultimate concern in people, such as cancer, are of concern at some level, say 10^{-4} to 10^{-6}, that is very low relative to the "normal" incidence for the specific disease, an example being lung cancer. In the case of laboratory animals or *in vitro* cell or tissue systems, the range of effects that may be observed is much more substantial.

This point should be kept in mind in the design and interpretation of studies, especially those using sub-animal systems. It is my contention that such studies should use a range of exposures (or doses) such that the range of effects varies down to a "background" incidence. This is especially the case if, as I believe it is, our ultimate goal is to understand the extent to which the changes in the cell system are causally related to the disease of interest in man at low levels of exposure. It must also be kept in mind that the increase in disease may result from some interaction between the factors causing the background incidence of the disease and toxicant induced changes.

Turning to the horizontal axis for which two scales are shown; one for the concentration of particles in air and the second for the concentration of a specific constituent. In this case, the specific constituent has arbitrarily been shown as being present at 10^{-5} of the total particle mass. This is not an unreasonable assumption, for example, for a specific organic compound present in particles of a complex mixture. Frequently in studying complex mixtures like diesel exhaust or cigarette smoke condensate, specific constituents will be separated from the mixture and studied in a less complex matrix or more frequently alone. When this is done, it is readily apparent that the exposure scale over which extrapolation must be made is further extended.

To assist in understanding Figure 5, let us consider a specific example - diesel exhaust. One concern for diesel exhaust focuses on environmental exposures of people which are likely to be on the order of 10 $\mu g/m^3$ or less. This is illustrated with the horizontal bar labeled "People." A question mark is shown on the vertical bar indicating our lack of knowledge of whether such exposures cause an excess of cancer in people. Turning next to whole animal exposures, the horizontal bar illustrates the range of concentrations of diesel soot that have been studied while the vertical bar illustrates the range of effects observed. In considering the exposure of laboratory animals, it should be kept in mind that the exposures are typically carried out for 6-20 hours per day for up to 2-1/2 years. The exposure or dosing of cell culture systems or the injection of animals is quite different, typically involving the delivery of a bolus of material in a very brief period of time. There is a substantial gradient in exposure rate (or dose rate) as one progresses from exposure of people, to inhalation exposure of animals to injection of animals and to dosing of cell cultures.

This gradient has significant implications for the design and interpretation of studies directed toward understanding and assessing human health risks at low exposure rates. It is reasonable to expect that some components of the processes of injury and repair will depend on both exposure rate and the total particle burden present at any given time. For example, the response of macrophages is likely to differ whether the macrophages are exposed to the same number of particles during the course of a few minutes or a few weeks. Furthermore, it is likely that the response of the macrophages and other cells will also depend on the total amount of particulate material present. In the same vein, the ability of cells to repair free radical damage to DNA is likely to be influenced by whether the free radical damage occurs over a few minutes or a few weeks.

For some time some individuals have advocated the use of a "maximum tolerated dose" (MTD) as an upper anchor exposure level in chronic bioassay studies. Traditionally, the MTD has been defined as a level of exposure that produces only modest levels of toxicity in the treated animals but does not substantially depress the body weight or reduce the life span of the exposed animals. The difficulties of identifying such an exposure level for inhalation studies has previously been addressed by an advisory group to the National Toxicology Program (Lewis et al., 1989). The occurrence of an overload condition in inhalation studies would appear to be a special kind of MTD phenomena.

Particle Overload Condition

The overload condition described at the beginning of this summary has now been described for several inhalation studies with different materials as related in this symposium. The nature of the condition is shown in Figure 6. The cycle of increased lung burden and pulmonary pathology is not well-understood today.

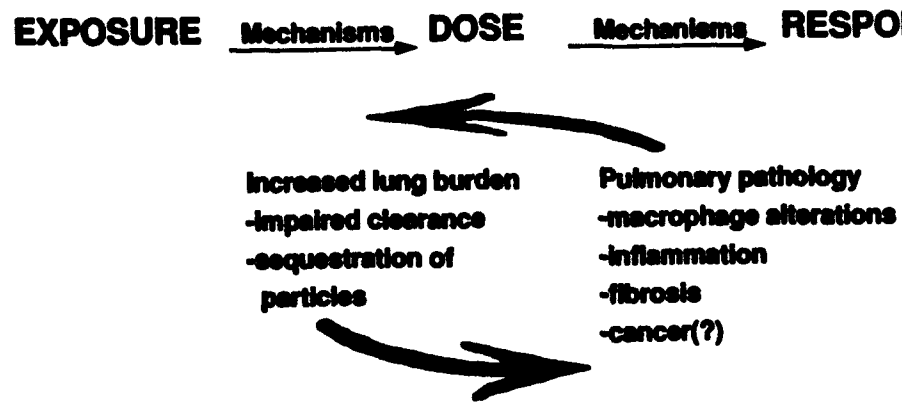

Figure 6. Schematic representative of the overload condition influencing both exposure-dose and dose-response relations.

Suffice it to say, though, that both components build on each other. The definition of an overload condition, by both the presence of increased lung burden, relative to that expected from disposition kinetics at lower exposure levels, and the presence of pulmonary pathology, puts a degree of stringency on the definition so that only a few inhalation studies can be judged relative to the definition. An example of a study fully meeting the criteria was the long term study of the carcinogenicity of diesel exhaust in rats conducted at the Lovelace Inhalation Toxicology Research Institute (Mauderly et al., 1987; Wolff et al., 1987; McClellan et al., 1986; McClellan, 1986).

The major shortcoming in most previous inhalation studies is a lack of quantitative information on the lung burden of test material as a function of exposure concentration and exposure time. I strongly suspect that if lung burden data were available on previous inhalation studies with particulate material, a number of them would meet the overload criteria I previously described. Obviously, nothing can be done with regard to recreating past studies. The challenge is to see that future inhalation studies with particulate material and fibers be conducted in a manner that permits an assessment of whether an overload condition develops. Appropriate guidance for designing such studies is contained in Lewis et al., 1989.

Unfortunately, our present level of knowledge of the overload phenomena does not allow us to separate the contribution of compound-specific versus non-compound specific effects in creating the overload condition. Most frequently, it is implied that non-compound specific effects are dominating the overload condition. Of particular concern is the development of fibrosis and lung cancer. A major unresolved issue is the extent to which cytokines may be influencing the development of fibrosis and, potentially, lung cancer (Kelly, 1990; Elias et al., 1990; Wahl et al., 1989; Agelli and Wahl, 1986; Kunkel et al., 1989).

Understanding the relative contribution of exposure concentration/non-compound specific effects versus compound specific toxicity in creating the overload condition is key to understanding the implications for use of the rat bioassay results for estimating human health risks at low exposure concentrations. This issue is depicted graphically in Figure 7 for two materials that have been extensively studied, quartz and TiO_2. In this schematic rendering, the specific toxicity of quartz is depicted as dominating its observed toxicity. In contrast, with TiO_2 the nonspecific effects are shown as dominant and thus mask any toxicity that might be specifically attributed to the TiO_2. A real dilemma occurs when both specific and non-specific effects occur at the same exposure concentrations and the observed effects are in fact interactive. Unfortunately, our present understanding of the overload condition is such that it is not clear as to how often the observed lung pathology is related to such interactions (Fig. 8).

Our lack of understanding of the overload condition is understandable when it is recognized that our present knowledge has largely been acquired from the

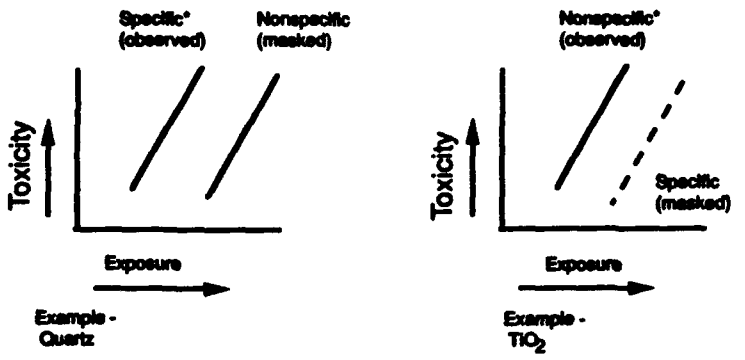

FIGURE 7. Interrelationship between compound specific and nonspecific effects.

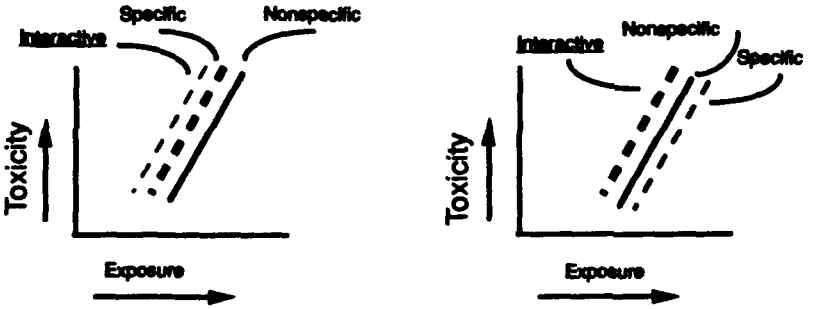

FIGURE 8. Interrelationship between compound specific and nonspecific effects.

opportunistic use of data from long-term studies designed for other purposes, i.e. to assess the carcinogenicity of the test material. In my opinion, the opportunity now exists to conduct hypothesis-testing investigations that are designed to evaluate the relative role of multiple factors in causing the overload condition. This includes consideration of

(1) exposure concentration and exposure time
(2) mass of deposited material
(3) clearance kinetics (physical removal, dissolution and translocation)
(4) particle characteristics (size, number, surface area, volume, parameter distribution)
(5) compound-specific toxicity
(6) specific differences and similarities which may be substantial in view of marked species differences in deposition and clearance of particles, and
(7) relationship between micro and macro-scale events, i.e. does local overload precede organ overload?

Design of Future Studies

At this juncture, I would like to comment specifically on the design of two types of studies, the standard cancer or toxicity assay and hypothesis-testing investigations. Both need to be designed, conducted, and interpreted with an exposure-dose-response orientation. In the past, cancer or toxicity bioassays typically did not give adequate consideration to characterization of dose. I strongly advocate that dose characterization be given a high priority from the earliest stage of the bioassay effort. This should include obtaining disposition data in 14 day or shorter term studies so it can be used in selecting exposure levels for 14 day, then 90 day and, ultimately, the 2 to 2-1/2 yr bioassay. The range of exposure concentrations selected for the long-term study should include one level expected to produce an overload condition (without such a level we will not know the relationship of the highest level used to a level that would produce

overload) and two or more levels that are not expected to produce an overload condition. Obviously, the study must include observations over a period of time approaching the life span of the animals. I personally advocate the maintenance of F344 rats for 2-1/2 years or until 20% of the animals survive, recognizing that close observation and removal of morbid animals allows for the prompt necropsy of most of the animals.

Hypothesis-testing investigations need to have an exposure-dose-response orientation with adequate attention given to dose characterization if the results are to have a high likelihood of being useful for assessing human risk. They should be clearly focused and designed to answer specific questions.

Putting Bioassay Study Results in Perspective

A major use of the results of bioassay studies is in the hazard identification and exposure-dose-response characterization elements of risk assessment. It is important to recognize that the exposure-dose-response characterization is ultimately brought together with an exposure characterization to give a risk characterization. In this context, it is important to recognize the importance of understanding how the test atmosphere used in the animal studies and the resultant lung burdens of the laboratory animals relate to the human exposure situation. This is shown in Figure 9. Several points can be made from the figure. First, it is important to recognize that the test atmosphere may not always mimic either the atmospheres likely to be encountered in the workplace or environmental settings. Frequently, test material may be substantially altered by grinding or size separation to maximize the respirable fraction in the test atmosphere and, thus, the detection of potential hazard. This must be kept in mind when interpreting the results. Second, the estimated human lung burden for various exposure situations, i.e. the likely workplace or environmental atmosphere versus the test atmosphere can be compared to the measured lung burden in the laboratory animals to put the bioassay results in perspective. In a sense, this sets aside potential species differences in exposure-dose relationships and allows one to focus on the dose-response relationship.

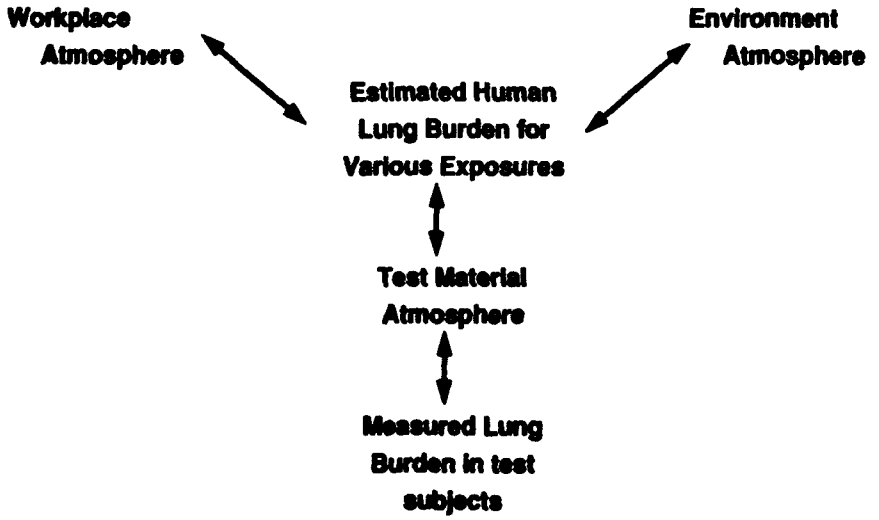

FIGURE 9. Important to put bioassay study results in perspective.

Need for Caution in Using Non-Physiological Modes of Administration

Some individuals have advocated the use of intratracheal instillation or intracavity injection as a mode of administering particles or fibers to assess

their toxicity and carcinogenicity. I have a high degree of concern for the use of these modes of administration, since with them an overload is created instantaneously. Likewise, the pathobiology of the overload condition is created in a matter of hours compared to what develops over a matter of months even with inhalation exposures at extraordinarily high levels. In my opinion, this significantly complicates the evaluation of compound-specific versus nonspecific toxicity resulting in a high likelihood that nonspecific overload effects may mask compound specific effects or that nonspecific overload effects may be erroneously characterized as being compound-specific.

In my opinion, extreme caution should be exercised in using data obtained from studies with non-physiological modes of administration for assessing human health risks for non-overload situations where compound specific toxicity is of greatest concern. I also urge caution in using non-physiological modes of administration for studying the pathogenesis of disease processes since the mechanisms being studied may be dominated by the overload condition and not be relevant to the situation likely to occur in laboratory animals or people with inhalation of low concentrations of material.

SUMMARY

The lung has a limited range of mechanisms of responding to inhaled toxicants. These mechanisms may be elicited both by characteristics of specific compounds and in a nonspecific manner. An understanding of both compound-specific toxicity and nonspecific toxicity and their relative importance at each level of exposure is crucial to the extrapolation of data from inhalation studies using laboratory animals to people and from one exposure situation to another.

The use of an exposure-dose-response orientation will aid in designing and interpreting inhalation studies using laboratory animals so the results will be useful for interpreting human health risks. A key consideration is the evaluation of the dose term to assess how it is influenced by exposure concentration and time. Further, it is crucial that information be obtained and integrated from various levels of biological organization, molecules to the intact animal, to understand the pathobiology of the overload phenomena.

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